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H 10019 PCT

Novel Use Of Adenoviruses and Nucleic Acids That Code For Said Viruses

The present invention relates to the use of adenoviruses as well as to nucleic acids coding therefor and recombinant viral oncoprotein.

A number of therapeutic concepts are currently used in the treatment of tumors. Apart from using surgery, chemotherapy and radiotherapy are predominant. All these techniques are, however, associated with considerable side effects. The use of replication selective oncolytic viruses provides for a new platform for the treatment of tumors. In connection therewith a selective intratumor replication of a viral agent is initiated which results in virus replication, lysis of the infected tumor cell and spreading of the virus to adjacent tumor cells. As the replication capabilities of the virus is limited to tumor cells, normal tissue is spared from replication and thus from lysis by the virus.

For the time being, several viral systems are subject to clinic trials aiming at tumor lysis. One example for such an adenovirus is dl1520 (Onyx-015) which has been successfully used in clinical phases I and II (Khuri, F. et al. Nature Medicine 6, 879-885, 2000). Onyx-015 is an adenovirus having a completely deleted E1B-55kDa gene. The complete deletion of the E1B55kDa protein of the adenovirus is based on the discovery that replication and thus lysis of cells is possible with an adenoviral vector having a p53 deficiency (Kirn, D. et al., Proc. Am. Soc. Clin. Oncol. 17, 391a, 1998), whereby normal cells are not harmed. More particularly, the E1B-55kDa gene product is involved in the inhibition of p53, the transport of viral mRNA and the switching off the protein synthesis of the host cell. The inhibition of p53 occurs via formation of a complex consisting of p53 and the adenoviral coded E1B-55kDa protein and/or a complex consisting of E1B-55kDa and E4orf6. p53, coded by TP53, is the starting point for a complex regulatory mechanism (Zambetti, G.P. et al., FASEB J. 7, 855-865, 1993), which results, among others, in an efficient inhibition of the replication in the cell of viruses like adenovirus. The gene TP 53 is deleted or mutated in about 50 % of all human tumors which results in the absence of – desired – apoptosis due to chemotherapy or radiation therapy resulting in an usually unsuccessful tumor treatment.

A further concept of tumorlytic adenoviruses is based on the discovery that if the E1A protein is present in a specific deleted form or comprises one or several mutations, which do not affect the binding of Rb/E2F and/or p107/E2F and/or p130/E2F, such adenovirus will not induce the entry of the infected cells into the S phase and will be capable of replicating in tumor cells which do not have a functional Rb protein. Additionally, the E1A protein can be deleted at the N-terminus and comprise one or several mutations in the region of amino acid positions 1 to 76 of the E1A proteins, respectively, in order to inhibit the binding of E1A to p300 and thus to provide for a selective replication in tumor cells. These approaches are described in an exemplary manner in European patent EP 0 931 830. Examples for such viruses are AdΔ24, dl922 – 947, E1Ad/01/07 and CB016 (Howe, J. A. et al., Molecular Therapy 2, 485-495, 2000; Fueyo, J. et al., Oncogene 19, 2-12, 2000; Heise, C. et al., Nature Medicine 6, 11341139, 2001; Balague, C. et al., J. Virol. 75, 7602-7611, 2001). These adenoviral systems for oncolysis known in the prior art thus comprise distinct deletions in the E1A protein, whereby such deletions had been made under the assumption that a functional Rb protein and complexes consisting of inactive Rb protein and E2F, respectively, would block an efficient in vivo replication and in order to provide an adenoviral replication in vivo in Rb-negative/mutated cells only. These adenoviral systems according to the prior art are based on E1A in order to control in vivo replication using the early E2 promoter (engl. E2 early promoter) and free E2F (Dyson, N. Genes & Development, 12, 2245-2262, 1998).

A further form of tumorlytic adenoviral systems is based on the use of selective promoters for specifically expressing the viral oncogene E1A which provides for a selective replication in tumor cells (Rodriguez, R. et al., Cancer Res. 57, 2559-2563, 1997).

As described above, the selection of a cellular background which is appropriate for the respective concept underlying the mode of action is important for the various concepts of adenoviral tumorlytic viruses. In other words, the various adenoviral systems currently known may only be used if distinct molecular biological prerequisites are realized. This limits the use of such systems to distinct patient groups.

A particular problem in the treatment of tumor diseases arises once the patients develop a so-called multidrug resistance (engl. multidrug resistance (MDR)) which represents a particularly well studied form of resistance of tumors against cytostatics (Gottesman and Pastan, Annu. Rev. Biochem. 62, 385-427, 1993). It is based on the overexpression of the membrane-bound

transport protein P-glycoprotein which belongs to the so-called ABC transporters (Stein, U. et al., JBC 276, 28562-69, 2001, J. Wijnholds, Novartis Found Symp., 243, 69-79, 2002). Bargou, R. C. et al. and Oda, Y. et al (Bargou, R. C. et al., Nature Medicine 3, 447-450, 1997; Clin. Cancer Res. 4, 2273-2277, 1998) were able to show that nuclear localisation of the human transcription factor YB-1 is directly involved in the activation of the expression of the P-glycoprotein. Further studies confirmed that YB-1 is transported into the nucleus by various stress conditions such as UV irradiation, administration of cytostatics (Koike, K. et al., FEBS Lett 17, 390-394, 1997) and hyperthermia (Stein, U. et al., JBC 276, 28562-69, 2001). Further studies confirmed that the nuclear localisation of YB-1 has an impact on one further ABC transporter. This ABC transporter is referred to as MRP (engl. multidrug resistance-related protein) and is involved in the formation of the so-called atypical non-P-glycoprotein dependent multidrug resistance (Stein, U. et al., JBC 276, 28562-69, 2001).

The problem underlying the present invention is to provide a technical teaching and in particular a means which allows to treat an organism, more particularly a human organism and a group of patients, respectively, specifically with tumorlytically active agents. It is a further problem underlying the present invention to provide a means which is suitable to cause tumorlysis in patients having tumor diseases which are resistant to cytostatics, particularly those which have a multidrug resistance.

According to the present invention the problem is solved in a first aspect by the use of a virus, preferably an adenovirus, for the manufacture of a medicament, whereby the virus is replication deficient in cells which do not have YB-1 in the nucleus, and the virus codes for an oncogene or oncogene product, preferably an oncogene protein, which transactivates at least one viral gene in YB-1 nucleus positive cells, preferably an adenoviral gene, whereby the gene is selected from the group comprising E1B55kDa, E4orf6, E4orf3 and E3ADP.

In a second aspect, the problem is solved by the use of a virus, preferably an adenovirus, for the replication in cells which have YB-1 in the nucleus, whereby the virus is replication deficient in cells which do not have YB-1 in the nucleus and the virus codes for an oncogene or oncogene product, in particular oncogene protein, which transactivates at least one viral gene, preferably an adenoviral gene, whereby the gene is selected from the group comprising E1B55kDa, E4orf6, E4orf3 and E3ADP.

In an embodiment of the two uses according to the invention, the virus, preferably the adenovirus, replicates in cells which have YB-1 in the nucleus.

In a further embodiment of the two uses according to the invention the viral oncogene protein is E1A and/or the oncogene is the gene coding for E1A and/or the oncogene protein is E1A.

In a preferred embodiment the viral oncogene protein E1A is capable of binding to a functional Rb tumor suppressor gene product.

In an alternative embodiment the viral oncogene protein E1A is not capable of binding to a functional Rb tumor suppressor gene product.

In a further embodiment of the two uses according to the invention the viral oncogene protein E1A is not inducing nuclear localisation of YB-1.

In a still further embodiment of the two uses according to the invention the medicament is for patients the cells of whom are either Rb-positive or Rb-negative.

In a preferred embodiment the cells are those cells which are involved in the formation of the condition which is to be influenced by the medicament.

In a further embodiment of the two uses according to the invention the cells are Rb-negative and are YB-1 positive in the nucleus, preferably are YB-1 positive in the nucleus independent from the cell cycle.

In a still further embodiment of the two uses according to the invention the medicament is for the treatment of tumors.

In a still further embodiment of the two uses according to the invention the cells, particularly the cells forming the tumor or parts thereof, are resistant to drugs, in particular have a multidrug resistance, preferably a resistance against anti-tumor agents and more preferably against cytostatics.

In a preferred embodiment of the two uses according to the invention the cells are expressing, preferably overexpressing the membrane-bound transport protein P-glycoprotein and/or MRP.

In a further embodiment of the two uses according to the invention the cells are p53-positive or p53-negative.

In an embodiment of the two uses according to the invention the oncogene protein has, compared to the wildtype oncogene protein E1A, one or several mutations or deletions, whereby the deletion is preferably selected from the group comprising deletions of the CR3 region and deletions of the N-terminus and deletions of the C-terminus. In connection therewith it is preferred that the E1A oncogene protein can bind to Rb.

In a further embodiment of the two uses according to the invention the oncogene protein has, compared to the wildtype oncogene protein, one or several mutations or deletions, whereby the deletion is preferably in the CR1 region and/or the CR2 region. It is within the invention that the oncogene protein E1A is incapable of binding to Rb.

In an embodiment of the two uses according to the invention the viral oncogene protein, in particular E1A, is under the control of a tissue-specific and/or tumor-specific promoter and/or an E1A promoter, in particular the natural E1A promoter.

In a further embodiment of the two uses according to the invention, the virus, in particular the adenovirus, codes for YB-1.

In a still further embodiment of the two uses according to the invention, YB-1 is under the control of a tissue-specific and/or tumor-specific promoter.

In a preferred embodiment of the two uses according to the invention, the virus, in particular the adenovirus, codes at least for one protein which is selected from the group comprising E4orf6, E4orf3, E1B55K and adenoviral E3ADP protein.

In an alternative embodiment of the two uses according to the invention, the cells have YB-1 in the nucleus, in particular the cells forming the tumor or part thereof have YB-1 in the nucleus.

In a further embodiment of the two uses according to the invention, the tumor has YB-1 in the nucleus upon inducing the transport of YB-1 into the nucleus.

In a preferred embodiment of the two uses according to the invention, the transport of YB-1 into the nucleus is triggered through at least one measure selected from the group comprising radiation, administration of cytostatics and hyperthermia.

In a particularly preferred embodiment of the two uses according to the invention, the measure is applied to a cell, an organ or an organism.

In a preferred embodiment of the two uses according to the invention, the virus, in particular the adenovirus, is selected from the group comprising AdΔ24, dl922-947, E1Ad/01/07, dl1119/1131, CB 016, dl520 and viruses which are lacking an expressed viral E1A oncogene which is capable of binding a functional Rb tumor suppressor gene product.

In a third aspect the problem is solved by the use of a virus, preferably an adenovirus, for the manufacture of a medicament, whereby the virus, preferably the adenovirus, is designed such that the replication is controlled through or by means of YB-1 through the activation of the E2-late promoter, preferably predominantly through the activation of the E2-late promoter. In an embodiment YB-1 is either a transgenic YB-1 or a cellular, in particular cellular deregulated YB-1. A transgenic YB-1 is preferably meant to be a YB-1 which is expressed in a cell by a vector, preferably a or the adenovirus. The E2-late promoter is preferably the adenoviral E2-late promoter as present in the wildtype adenovirus, or an E2-late promoter as described herein in connection with the expression of transgenes.

In a fourth aspect the problem is solved by the use of a virus and particular an adenovirus, for the replication in cells which have YB-1 in the nucleus, whereby the virus, in particular the adenovirus, is designed such that the replication is controlled by YB-1 through the activation of the E2-late promoter, preferably predominantly through the activation of the E2-late promoter. In an embodiment YB-1 is either a transgenic YB-1 or a cellular, in particular cellular deregulated YB-1. A transgenic YB-1 as used herein is preferably a YB-1 which is expressed in a cell by a vector, preferably a or the adenovirus. The E2-late promoter is

preferably the adenoviral E2-late promoter as present in the wildtype adenovirus, or an E2-late promoter as described herein in connection with the use of the expression of transgenes.

In a preferred embodiment of the third and/or fourth aspect of the present invention the adenovirus is designed such as disclosed herein, particularly such as it is designed in order to be used in accordance with the present invention.

In a fifth aspect the problem is solved by a viral oncogene protein, in particular an isolated viral oncogene protein which has the following characteristics:

a) transactivation of at least one viral gene in YB-1 nucleus-positive cells, which is selected from the group comprising E1B-55K, E3ADP and E4orf6 and E4orf3; and

b) lacking induction of YB-1 in the nucleus, in particular in the nucleus of the cell in which the viral oncogene protein is present.

In an embodiment the viral oncoprotein is E1A.

In a further embodiment the viral oncogene protein has, compared to the wildtype oncogene protein, one or several mutations or deletions, whereby the deletion is preferably selected from the group comprising deletion of the CR3 region, deletion of the N-terminus and deletion of the C-terminus.

In an embodiment the induction of YB-1 through the viral oncogene protein is absent when E4orf6 and/or E1B 55 kD is/are not present in the nucleus exhibiting cell.

In connection therewith it is intended that the viral oncogene protein is capable of binding to Rb.

In an alternative embodiment the viral oncogene protein comprises one or several mutations or deletions, whereby the deletion is preferably in the CR1 region and/or the CR2 region of the E1A oncogene protein. In connection therewith it is intended that the viral oncogene protein is not able to bind to Rb.

In a sixth aspect the invention is related to the use of a viral replication system, preferably an adenoviral replication system, comprising a nucleic acid which codes for a virus, in particular an adenovirus as used in accordance with the present invention, and comprising one nucleic acid of a helper virus, whereby the nucleic acid of the helper virus comprises a nucleic acid which codes for YB-1.

In an embodiment the viral nucleic acid, in particular the adenoviral nucleic acid, and/or the nucleic acid of the helper virus are present as a vector which can replicate.

In a seventh aspect the invention is related to the use of a nucleic acid coding for a virus, in particular an adenovirus, as it is used in accordance with the invention, for the manufacture of a medicament, in particular for the manufacture of a medicament for the treatment of tumors.

In an embodiment the cells, in particular the cells forming the tumor or parts thereof, are resistant, in particular have a multidrug resistance, against drugs, preferably anti-tumor agents, and more preferably cytostatics.

In an eighth aspect the invention is related to the use of a nucleic acid which codes for a virus, in particular an adenovirus, as is used in accordance with the present invention, for the replication in cells which have YB-1 in the nucleus, whereby the virus is replication deficient in cells which do not have YB-1 in the nucleus, and the virus codes for an oncogene or oncogene product which transactivates at least one viral gene, preferably an adenoviral gene, in YB-1 nucleus-positive cells, whereby the gene is selected from the group comprising E1B55kDa, E4orf6, E4orf3 and E3ADP.

In a ninth aspect the problem is solved by the use of a nucleic acid which codes for a virus, preferably an adenovirus, as is used in accordance with the invention, for the manufacture of a medicament, whereby the virus is designed such that the replication is controlled by YB-1 through the activation of the E2-late promoters, preferably predominantly through the activation of the E2-late promoter. In an embodiment the YB-1 is either a transgenic YB-1 or a cellular, in particular cellular deregulated YB-1. A transgenic YB-1 as used herein is preferably a YB-1 which is expressed in a cell by a vector, preferably a or the adenovirus. The E2-late promoter is preferably the adenoviral E2-late promoter as is present in the wildtype

adenovirus, or an E2-late promoter as described herein in connection with the use of the expression of transgenes.

In a tenth aspect the problem is solved by the use of a nucleic acid which codes for a virus, in particular an adenovirus, as used in accordance with the invention for replication in cells, whereby the virus is designed such that the replication is controlled by YB-1 through the activation of the E2-late promoter, preferably predominantly through the activation of the E2-late promoter. In an embodiment the YB-1 is either a transgenic YB-1 or a cellular, in particular cellular deregulated YB-1. As used herein, transgenic YB-1 is preferably a YB-1 which is expressed by a vector in a cell, preferably by a or the adenovirus. The E2-late promoter is preferably the adenoviral E2-late promoter as present in wildtype adenovirus, or an E2-late promoter as used in connection with the expression of transgenes described herein.

In an eleventh aspect the problem is solved by the use of a vector comprising one of the previously described nucleic acids, for the use in accordance with the first or second aspect of the present invention.

In a twelfth aspect the invention is related to the use of an agent interacting with YB-1 for the characterisation of cells, cells of a tumor tissue or patients, in order to determine whether these shall be contacted and/or treated with a virus, in particular an adenovirus, which is used in accordance with the invention.

In an embodiment the agent is selected from the group comprising antibodies, anticalines, aptamers, aptazymes and spiegelmers.

In a thirteenth aspect the problem is solved by the use of the viral oncogene protein according to the present invention or a nucleic acid coding therefor, for the manufacture of a virus, in particular an adenovirus, which is used in accordance with the first and second aspect of the present invention.

In an embodiment the virus comprises a nucleic acid coding for a transgene.

In a further embodiment the virus comprises the translation product and/or the transcription product of a transgene.

In a preferred embodiment the nucleic acid of the adenoviral replication system and/or the nucleic acid of the helper virus comprises a transgene or a nucleic acid coding for a transgene.

In a still further embodiment the nucleic acid comprises a transgene or a nucleic acid coding for a transgene.

In an alternative embodiment the transgene is selected from the group comprising prodrug genes, cytokines, apoptosis-inducing genes, tumor suppressor genes, genes for metalloproteinases inhibitors and genes for angiogenesis inhibitors.

In an embodiment the transgene is selected from the group comprising nucleic acids for siRNA, for aptamers, for antisense molecules and for ribozymes, whereby the siRNA, the aptamers, the antisense molecules and/or the ribozymes are targeted against a target molecule.

In a further embodiment the target molecule is selected from the group comprising resistance relevant factors, anti-apoptosis factors, oncogenes, angiogenesis factors, DNA synthesis enzymes, DNA repair enzymes, growth factors and their receptors, transcription factors, metalloproteinases, in particular matrix metalloproteinases, and plasminogen activator of the urokinase type. In an embodiment the resistance-relevant factors are preferably selected from the group comprising P-glycoprotein, MRP and GST, and also comprise nucleic acids coding therefor. In an embodiment the anti-apoptosis factors are selected from the group comprising BCL2, and also comprise the nucleic acids coding therefor. In an embodiment the oncogenes are selected from the group comprising Ras, in particular mutated Ras, Rb and Myc, and also comprise nucleic acids coding therefor. In an embodiment the angiogenesis factors are selected from the group comprising VEGF and HMG proteins and also comprise the nucleic acids coding therefor. In an embodiment the DNA synthesis enzymes are selected from the group comprising telomerase and also comprise nucleic acids coding therefor. In an embodiment the DNA repair enzymes are selected from the group which comprises Ku-80, and also comprise nucleic acids coding therefor. In an embodiment the growth factors are selected from the group comprising PDGF, EGF and M-CSF, and comprise also nucleic acids coding therefor. In an embodiment the receptors are in particular receptors for growth factors, whereby the growth factors are preferably selected from the group comprising PDGF, EGF and M-CSF, and also comprise the nucleic acids coding therefor. In an embodiment the

transcription factors are selected from the group comprising YB-1, and also comprise the nucleic acid coding therefor. In an embodiment the metalloproteinases are preferably matrix metalloproteinases. In a preferred embodiment the matrix metalloproteinases are selected from the group comprising MMP-1 and MMP-2, and also comprise the nucleic acids coding therefor. In an embodiment the plasminogen activators of the urokinase type are selected from the group comprising uPa-R, and also comprise the nucleic acids coding therefor.

In a still further embodiment the medicament comprises additionally at least one pharmaceutically active compound.

In a preferred embodiment the pharmaceutically active compound is selected from the group comprising cytokines, metalloproteinase inhibitors, angiogenesis inhibitors, cytostatics such as Irinotecan and CPT-11 against colorectal carcinoma and Daunorubicin against leukemia, cell cycle inhibitors such as CYC202 which inhibits CDK2/CyclinE kinase activity and can be used against colorectal tumors (McClue SJ, Int. J. Cancer 2002, 102, 463-468) and BAY 43-9006 which inhibits Raf-1 and is, for example, effective against mamma carcinoma (Wilhelm SM et al., Cancer Res. 2004, 64, 7099-7109), proteosome inhibitors such as PS-341 which inhibits the 26S proteasome activity and is used against squamous-cell carcinoma (Fribley A et al., Mol Cell Biol 2004 Nov; 24(22): 9695-704), recombinant antibodies such as against the EGF receptor (Herceptin for breast carcinoma and prostate tumor; H.G. van der Poel, European Urology 2004, 1-17; Erbitux against head and neck tumors; Bauman M et al., Radiother. Oncol., 2004, 72, 257-266), and inhibitors of the signal transduction cascade such as STI 571 which represses, among others, c-kit and can be used against gastrointestinal tumors (H.G. van der Poel, European Urology 2004, 45, 1-17), ABT-627, an endothelin inhibitor, which may be used, among others, against prostate tumors (H.G. van der Poel, European Urology 2004, 45, 1-17), SU5416 which inhibits phosphorylation of the VEGF tyrosine kinase receptor and which may be used, among others, against glioblastoma and prostate cancer (Bischof M et al Int. J. Radiat. Oncol. Biol. Phys. 2004; 60 (4): 1220-32), ZD1839 which inhibits EGFR tyrosine activity and may be used, among others, against prostate tumors (H.G. van der Poel, European Urology 2004, 45, 1-17); rapamycin derivatives such as CCI-779 and RAD001 which inhibit mTOR and can be used against prostate tumors. It is within the present invention that the various adenoviruses described herein and the adenoviruses to be used in accordance with the present invention, respectively, can, in principle, be used with each and any of the aforementioned compounds for each and

any of the indication described herein in connection therewith. In a particularly preferred embodiment the indication is the one which is described for any of the previously mentioned pharmaceutically active compounds.

In an embodiment the medicament comprises a combination of at least two agents, whereby any agent is individually and independently selected from the group comprising cytostatics.

In a preferred embodiment at least two of the agent act upon different target molecules.

In an alternative embodiment at least two of the agents are active through a different mode of action.

In an embodiment at least one agents increases the capacity of a cell to be infected in which the virus replicates.

In an embodiment at least one agent has an impact on the availability of a component within the cell, which preferably increases the availability of the component, whereby the component mediates the uptake of the virus.

In an embodiment at least one agent mediates the transport inof YB-1 to the nucleus, preferably increases said transport.

In an embodiment at least one agent is a histone deacylase inhibitor.

In a preferred embodiment the histone deacylase inhibitor is selected from the group comprising trichostatine A, FR 901228, MS-27-275, NVP-LAQ824 and PXD101.

In an embodiment at least one agent is selected from the group comprising trichostatin A (against glioblastoma, Kim JH et al., Int. J. Radiation Oncology Biol. Phys. 2004, 59, 1174-1180), FR 901228 (against pancreas tumors, Sato N et al., Int. J. Oncol. 2004, 24, 679-685; MS-27-275 (against prostate tumors; Camphausen K et al., Clinical Canver Research 2004, 10, 6066-6071), NVP-LAQ824 (against leukemiae; Nimmanapalli R et al., Cancer Res. 2003, 63, 5126-5135; PXD101 (against ovary tumors, Plumb JA et al, Mol. Cancer Ther. 2003, 2, 721-728), Scriptaid (against breast carcinoma, Keen JC et al., Breast Cancer Res. Treat. 2003,

81, 177-186), apicidin (against melanoma, Kim SH et al., Biochem. Biophys. Res. Commun. 2004, 315, 964-970) and CI-994 (against various tumors, Nemunaitis JJ et al., Cancer J. 2003, 9, 58-66). The mode of action of histone deacetylase inhibitors is, among others, described in Lindemann RK et al., Cell Cycle 2004, 3, 77-86. It is within the present invention that the various adenoviruses described herein and the adenoviruses to be used in accordance with the present invention may be used with the aforementioned compounds, in principle, for each and any of the indications described herein in connection therewith. In a particularly preferred embodiment the indication is one as has been described for each and any of the aforementioned pharmaceutically active compounds.

In an embodiment at least one agent is a topoisomerase inhibitor.

In a preferred embodiment the topoisomerase inhibitor is selected from the group comprising camptothecin, irinotecan, topotecan, DX-895If, SN-38, 9-aminocamptothecin, 9-nitrocamptothecin, etoposid and daunorubicin. These may be used against various tumors, for example, colorectal tumors, pancreas tumors, ovary carcinomas and prostate carcinomas. The fields of application are, among others, described by Recchia F et al., British J. Cancer 2004, 91, 1442-1446; Cantore M et al., Oncology 2004, 67, 93-97; Maurel J. et al., Gynecol. Oncol 2004, 95, 114-119; Amin A. et al., Urol. Oncol. 2004, 22, 398-403; Kindler HL et al., Invest. New Drugs 2004, 22, 323-327, Ahmad T. et al., Expert Opin. Pharmacother. 2004, 5, 2333-2340; Azzariti A. et al., Biochem Pharmacol. 2004, 68, 135-144; Le QT et al., Clinical Cancer Res. 2004, 10, 5418-5424. It is within the present invention that the various adenoviruses described herein and the adenoviruses to be used in accordance with the present invention, respectively, may in principle be used with the aforementioned compounds for each and any of the indications described herein in connection therewith. In a particularly preferred embodiment the indication is such as described for each of the aforementioned pharmaceutically active compounds.

In a particularly preferred embodiment the indication is one which is described for any of the afore mentioned pharmaceutically active compounds.

In a preferred embodiment the means comprises trichostatine A and irinotecan and daunorubicin.

In an embodiment the virus, in particular the virus in accordance with one of the aspects of the present invention, is separated from the at least two agents.

In a preferred embodiment at least one unit dosis of the virus is separated from at least one unit dosis of one or the at least two agents.

In a fourteenth aspect the invention is related to a kit comprising a virus, in particular a virus according to any aspect of the present invention, and at least two agents, whereby any agent is individually and independently selected from the group comprising cytostatics.

The present invention is based on the surprising finding that the DNA replication of E1A-modified adenoviruses in YB-1 nucleus-positive tumor cells is based on the activation of the E2-late promoter. E1A-modified adenoviruses as used herein, are adenoviruses which (a) do not replicate in YB-1 nucleus-negative cells or show a reduced, preferably a strongly reduced replication in YB-1 nucleus-negative cells compared to the respective wildtype, (b) transactivate at least one viral gene, whereby the gene is in particular selected from the group comprising E1B-55kDa, E4orf6, E4orf3 and E3ADP, and/or (c) do not translocate cellular YB-1 through the adenovirus into the nucleus. Optionally the adenoviruses used in accordance with the present invention have the further characteristic that the binding of the adenoviral encoded E1A protein interferes with the binding of E2F to Rb and is able to dissolve the respective complex consisting of E2F and Rb, respectively. Adenoviruses which have at least one or several of the aforementioned features a) to c), preferably all of features a) to c), are replication deficient in cells which do not have YB-1 in the nucleus.

In an embodiment a strongly reduced replication as used herein particularly means a replication which, compared to the wildtype, is reduced by a factor of 2, preferably by a factor of 5, more preferably by a factor of 10 and most preferably by a factor of 100. In a preferred embodiment such comparison of the replication is performed using identical or similar cell lines, identical or similar virus titers for infection (multiplicity of infection, MOI, or plaque forming unit, pfu) and/or identical or similar general experimental conditions. Replication as used herein particularly means formation of particles. In a further embodiment the measure for replication can be the extent of viral nucleic acid synthesis. Methods for the determination

of the extent of the viral nucleic acid synthesis as well as methods for determining particle formation are known to the ones skilled in the art.

The findings, methods, uses or nucleic acids, proteins, replication systems and the like described herein are not necessarily limited to adenoviruses. In principle, such systems exist also in other viruses which are herewith also encompassed.

A replication which is comparable to wildtype replication, can be realized upon an infection rate of 1 to 10 pfu/cell compared to 10 to 100 pfu/cell according to the prior art when using the viruses according to the present invention or when using the viruses described herein in accordance with the present invention.

Cellular YB-1 as used herein shall mean any YB-1 which is coded by a cell and preferably is also expressed by a cell, whereby this YB-1 is present in the cell, preferably prior to the infection of the respective cell with an adenovirus, preferably an adenovirus and/or a helpervirus as described herein. It is, however, also within the present invention that cellular YB-1 is a YB-1 which is introduced into the cell or produced by such cell upon application of exogenous measures such as, e. g., infection with a virus, in particular with an adenovirus.

Without wishing to be bound by this in the following, the present inventor assumes that the E2-early promoter, i. e. the early E2 promoter is not switched on through the human cellular E2F transcription factor in connection with the replication of the viruses used herein in accordance with the present invention. The switching on of the replication is independent of the Rb status of the cells, i. e. which means that the tumor cells which are infected using the viruses disclosed herein and which are preferably lysed subsequently thereafter, may comprise both functional as well as inactive Rb proteins. Additionally, adenoviral replication does neither need any functional p53 protein nor is it affected by its presence, when using the adenoviruses disclosed herein or under the conditions disclosed herein. Insofar, the technical teaching departs from the principle underlying the use of the oncolytic or tumorlytic adenoviruses of the AdΔ24, dl922-947, E1Ad/01/07, CB016 type or of those adenoviruses which are, for example, described in European patent EP 0 931 830, and into which one or several deletions have been introduced into the E1A protein under the assumption that intact functional Rb proteins are an obstacle to an efficient replication in vivo thus providing an adenoviral replication in vivo only in Rb-negative and Rb-mutated cells, respectively. These

adenoviral systems according to the prior art are based on E1A in order to control in vivo replication of adenoviruses by means of the early E2 promoter (E2 early promoter) and "free E2F". Nevertheless, these viruses according to the prior art may be used in accordance with the present invention, i. e. for replication in cells which contain YB-1 in the nucleus independent from the cell cycle.

The viruses described in said European patent EP 0 931 830 and in particular adenoviruses may be used in accordance with the present invention. More particularly, the viruses described in said patent are replication deficient and lack an expressed viral oncoprotein which is capable of binding a functional Rb tumor suppressor gene product. The adenovirus can particularly be an adenovirus which is lacking expressed viral E1A oncoprotein which is capable of binding a functional tumor suppressor gene product, in particular Rb. The viral E1A oncoprotein can comprise an inactivating mutation, for example in the CR1 domain at amino acid positions 30 to 85 in Ad 5, nucleotide positions 697 to 790 and/or the CR2 domain at amino acid positions 120 to 139 in Ad 5, nucleotide positions 920 to 967 which are involved in the binding of p105 Rb protein, p130 and p107 protein. It can also be intended that the adenovirus is of type 2 dl 312 or the adenovirus is of type 5 NT dl 1010.

Replication ultimately occurs in cells which comprise YB-1 in the nucleus, preferably independent from the cell cycle, which are thus YB-1 nucleus-positive, when using adenoviruses in accordance with the invention for the manufacture of a medicament, in particular for the manufacture of a medicament for the treatment of tumor diseases, and when using adenoviruses in accordance with the invention for replication in cells which have YB-1 in the nucleus. It is particularly noteworthy that the adenoviruses as such do not replicate in cells which do not have YB-1 in the nucleus but have YB-1 essentially in the cytoplasm only, or replicate at a significantly reduced level. Insofar it is necessary that YB-1 is present in the nucleus for a successful replication of these viruses. This can, for example, as will be outlined in the following in more detail, be realized by applying measures to the cells which result in the expression of YB-1 in the nucleus or in the presence of YB-1 in the nucleus. A respective measure can, for example, be the coding and expression, respectively, of YB-1 through adenoviruses used in accordance with the present invention which in addition to the adenoviral genes also comprise a genetic information coding for YB-1 and in particular for the expression of YB-1. Other measures which result in transport, induction or expression of

YB-1 in the nucleus of the cell, are stress conditions such as administration of cytostatics, irradiation, hyperthermia and the like, to the cell and to an organism containing such cell.

The adenoviruses which are used in connection with the present invention, in particular for tumor lysis, are further characterized such that they do not replicate in cells which do not have YB-1 in the nucleus, in other words which are YB-1 nucleus-negative.

A further feature of the adenoviruses which are to be used in accordance with the invention, is that they code for a viral oncoprotein which is also referred to herein as oncogene protein, whereby the oncogene protein is preferably E1A, whereby the oncogene protein is capable of activating at least one viral gene which can have an impact on the replication of the virus and/or cell lysis of the cells infected by the virus. It is preferred that the influence on replication is such that the virus replicates better in the presence of the oncogene protein compared to a situation where the oncogene protein of the respective virus is lacking. This process is referred to herein also as transactivating and in particular E1A transactivating, when the transactivation is mediated through E1A. The term "transactivate" or "transactivation" describes preferably the process that the respective viral oncoprotein has an impact on the expression and/or the transcription of one or several other genes different from the viral oncoprotein coding gene itself, i. e. is preferably controlling its expression and/or translation, and in particular activates this/these. Such viral genes are preferably E1B55kDa, E4orf6, E4orf3 and E3ADP as well as any combination of the aforementioned genes and gene products, respectively.

A further, although preferably optional, feature of the adenoviruses to be used in accordance with the invention, is the binding to and of tumor suppressor Rb. In principle it is within the present invention that the adenoviruses used in accordance with the present invention bind to Rb or do not bind to Rb. The use of both alternative embodiments of the adenoviruses is possible independently from the Rb status of the cell to be treated.

In order to confer the capability to not bind to Rb, the following deletions of the E1A oncoprotein are, for example, possible: Deletion in the CR1 region (amino acid positions 30 – 85 in Ad5) and deletion of the CR2 region (amino acid positions 120 – 139 in AD5). In doing so, the CR3 region is maintained and can have its transactivating function on the other early viral genes.

In contrast thereto, the following deletions to the E1A oncoprotein are in principle possible in order to impart E1A the capability to bind to Rb: deletion of the CR3 region (amino acid positions 140 – 185); deletion of the N-terminus (amino acid positions 1 – 29); deletion of amino acid positions 85 – 119; and deletion of the C-terminus (amino acid positions 186 – 289). The regions recited herein do not interfere with the binding of E2F to Rb. The transactivating function remains, however, is reduced compared to wildtype Ad5.

Such viruses which are known in the prior art are generally regarded as replication deficient. It is, however, the merit of the present inventor that he has recognised that they are capable of replication in a suitable background nevertheless, in particular a cellular background. Such a suitable cellular background is caused or provided by the presence of YB-1 in the nucleus, preferably a cell cycle independent presence of YB-1 in the nucleus. The term cells or cellular systems, as used herein, comprises fragments of cells or fractions of cell lysates as well as cells which are present in vitro, in vivo or in situ. Insofar, the term cellular systems or cells also comprises cells which are present in a cell culture, tissue culture, organ culture or in any other tissue or organ in vivo and in situ, respectively, isolated, in groups or as part of tissues, organs or organisms or are also present as such in a preferably living organism. The organism is preferably a vertebrate organism and more preferably a mammal. It is particularly preferred that the organism is a human organism.

Additionally, it is within the present invention that based on the technical teaching provided herein, new viruses are generated which have the replication characteristic of the adenoviruses described herein as well as the one of adenoviruses of the prior art in cells which are YB-1 nucleus-positive. In other words, preferably starting from the adenoviruses already known further viruses can be designed which have the features defined herein needed for their use in accordance with the present invention.

In connection with the present invention the modified E1A oncoprotein of the various adenoviruses which are to be used in accordance with the invention, is capable of transactivating the early viral genes such as, for example, E1B55K, E4orf3, E4orf6, E3ADP, in YB-1 nucleus-positive cells. In connection therewith, there are preferably otherwise no further changes to the viral genome and the respective adenovirus can otherwise correspond to an adenovirus of the wildtype or any derivative thereof.

The viruses disclosed herein which code for a transactivating oncogene protein in the sense of the present invention or which comprise such oncogene protein, comprise, for example, the adenoviruses AdΔ24, dl922-947, E1Ad/01/07, CB106 and/or the adenoviruses described in European patent EP 0 931 380, which are each capable of transactivating the early genes, such as E1B, E2, E3 and/or E4, and are comparable to adenoviruses of the wildtype, in particular wildtype Ad5. A particular region of the E1A protein is responsible for transactivation in these cases. Within various adenovirus serotypes there are three highly conserved regions in the E1A protein. The CR1 region from amino acid positions 41 – 80, the CR2 region from amino acid positions 120 – 139 and the CR3 region from of amino acid positions 140 – 188. The transactivating function is primarily based on the presence of the CR3 region in the E1A protein. The amino acid sequence of CR3 is unaltered in the aforementioned adenoviruses. This results in a transactivation of the early genes E1B, E2, E3 and E4 independent from the presence of YB-1 in the nucleus or in the cytoplasma.

In the recombinant adenovirus dl520, however, the CR3 region has been deleted. Thus dl520 expresses a so-called E1A12S protein which does not comprise the amino acid sequence of the CR3 region. As a consequence, dl520 can exert a very weak transactivating function only, in particular on the E2 region, and thus does not replicate in YB-1 nucleus-negative cells. In YB-1 nucleus-positive cells YB-1 is transactivating the E2 region and thus allows an efficient replication of dl520. This is the basis for the use of systems like dl520 and of systems on the basis of dl520 for the purposes disclosed herein, respectively. A further important difference between both the previously described groups of adenoviruses, i. e. delta 24 (herein also referred to as AdΔ24) and dl520 resides in the fact that with dl520 the early genes E1B, E3 and E4 are more strongly transactivated in YB-1 nucleus-positive cells compared to YB-1 nucleus-negative cells. In contrast, there are no or only minor differences with delta 24. The transactivation effect of dl520 and more particularly of the E1A12S protein, however, is significantly reduced compared to wildtype adenovirus. This transactivation is, however, sufficient in order to allow for an efficient replication in YB-1 nucleus-positive cells, as shown in example 10. The design of the E1A protein and of the nucleic acid coding therefor described herein and in particular in this context such that the E1A protein has one or several deletions and/or mutations compared to the wildtype oncogene protein E1A, whereby the deletion is preferably one selected from the group comprising deletions of the CR3 region and deletions of the N-terminus and deletions of the C-terminus, including and particularly

preferred those embodiments of the E1A protein as described in connection with dl520 or AdΔ24, dl922 – 947, E1Ad/01/07, CB106 and/or the adenoviruses described in European patent EP 0 931 830, are embodiments of viruses, in particular adenoviruses, the replication of which is controlled by YB-1 through the activation of the E2-late promoter, preferably predominantly through the activation of the E2-late promoter. Further embodiments of the E1A protein which allow this form of replication of adenoviruses, can be generated by the ones skilled in the art based on the disclosure provided herein.

In further adenoviruses which are to be newly constructed, which are also referred to herein as derivatives and which may be used in accordance with the present invention, typically have an E1 deletion, an E1/E3 deletion and/or an E4 deletion, i. e . the corresponding adenoviruses are not able to generate functionally active E1 and/or E3 and/or E4 expression products and respective products, respectively, or, in other words, these adenoviruses are only capable to generate functional inactive E1, E3 and/or E4 expression products, whereby a functionally inactive E1, E3 and/or E4 expression product as such which is either not present as an expression product at all, whether at the transcription level and/or the translation level, or it is present in a form in which it at least is lacking one of the functions it has in wildtype adenoviruses. The function(s) of the expression product of the wildtype adenovirus is/are known to the ones skilled in the art and, for example, described in Russell, W. C., Journal of Virology, 81, 2573-2604, 2000. Russell (supra) describes also principles for the construction of adenoviruses and adenoviral vectors which are incorporated herein by reference. It is also within the present invention that the modified E1A oncoprotein, E1B-55K, E4orf6 and/or E3ADP (adenoviral death protein (ADP)) (Tollefson, A. et al., J. Virology, 70, 2296-2306, 1996) is expressed in such a vector either individually or in any combination. In connection therewith, the individually named genes as well as the transgenes disclosed herein and/or the therapeutic transgenes, can be cloned into the E1 and/or E3 and/or E4 region and be expressed independently by virtue of a suitable promoter or under the control of a suitable promoter. Basically, the regions E1, E3 and E4 are similarly suitable as cloning sites within the adenoviral nucleic acid, whereby the regions not used for the cloning can, either individually or as a whole, be present, partially and/or completely deleted. In case that these regions are present, in particular are present in their entirety, it is within the present invention that they are either intact and preferably provide for a translation product and/or a transcription product, and/or are not intact and preferably do not provide for a translation product and/transcription product. Suitable promoters are, among others, those as disclosed herein in

connection with the control and expression, respectively, of E1A, in particular of the modified E1A.

Finally, in one embodiment the adenoviruses which are to be used in accordance with the present invention, are deficient with regard to E1B, in particular with regard to E1B 19 kDa. As used herein, the term deficient generally means a condition in which E1B does not have all of the characteristics inherent to the wildtype but at least one of these characteristics is absent. The adenoviral BCL2 homolog E1B19k avoids the E1A induced apoptosis by interaction with the pro-apoptotic proteins Bak and Bax. Because of this a maximum replication and/or particle formation is possible in infected cells (Ramya Sundararajan and Eileen White, Journal of Virology 2001, 75, 7506-7516). The lack of E1B19k results in a better release of the viruses as, if present, it will minimize the function of the adenoviral death protein. By such a deletion the virus induced cytopathic effect is increased (Ta-Chiang Liu et al., Molecular Therapy, 2004) and thus results in a more pronounced lysis of infected tumor cells. Additionally, the lack of E1B19k causes that TNF-alpha does not exert an influence on the replication of such recombinant adenoviruses in tumor cells, whereas in normal cells the treatment results in a reduced replication and release of infectious viruses. Insofar, selectivity and specificity are increased (Ta-Chiang Liu et al., Molecular Therapy 2004, 9, 786-803).

The adenoviruses which are used in accordance with the invention disclosed herein, are, basically, known in the prior art in some embodiments. The adenoviruses used in accordance with the present invention are preferably recombinant adenoviruses, particularly also when a change, compared to the wildtype, has been made in accordance with the technical teaching provided herein. It is within the skills of those of the art to delete or mutate those adenoviral nucleic acid sequences which are not essential for the present invention. Such deletions may, for example, be related to a part of the nucleic acid coding for E3 and E4 as also described herein. A deletion of E4 is particularly preferred if such deletion does not extend to the protein E4orf6, or, in other words, the adenovirus to be used in accordance with the present invention codes for E4orf6. In preferred embodiments these adenoviral nucleic acids may still be packed into the viral capsid and may thus form infectious particles. The same is true for the use of the nucleic acids in accordance with the present invention. It should be noted that in general the adenoviral systems may be deficient with regard to single or several expression products. In connection therewith it is to be taken into consideration that this may be either based on the fact that the nucleic acid coding for such expression product is completely

mutated or deleted or mutated or deleted to the extent that essentially no expression product is produced anymore or based on the lack of promoters or transcription factors which control the expression, or which are active in a manner different from wildtype, either at the nucleic acid level (lack of a promoter; cis-acting element) or at the translation system and the transcription system, respectively (trans-acting elements). Particularly the latter aspect may be dependent on the cellular background.

Apart from using adenoviruses in accordance with the present invention, which are already known, also novel adenoviruses can be used to the same extent as has already been disclosed for the other adenoviruses described herein. The novel adenoviruses according to the invention result from the technical teaching provided herein. Particularly preferred representatives are, for example, the viruses Xvir03 and Xvir03/01 depicted in Fig. 16 and Fig. 17, the design principle of which is also further illustrated in examples 11 and 12.

In the case of vector Xvir03 a CMV promoter is cloned into the E1 region which codes the nucleic acids for E1B 55K and E4orf6, which are separated by a IRES sequence. In connection therewith the E3 region may be partially or completely deleted and/or may be present in a completely intact form. Due to the introduction of these two genes and the gene products produced therefrom, respectively, a replication efficiency is created which factually corresponds to the one of wildtype viruses, whereby the selectivity of the replication is maintained for cells, particularly tumor cells, insofar as a replication happens in particular in YB-1 nucleus-positive cells and more particularly in cells in which YB-1 is deregulated. Cells in which YB-1 is deregulated, are preferably those which show an increased expression of YB-1, preferably compartment-independent, compared to normal or non-tumor cells. E1B 55k and E4orf6 can also be cloned into the E4 region, whereby the E3 region can be intact or/and partially or completely deleted.

A further development of virus Xvir03 is virus Xvir03/01 into which, in a preferred embodiment, therapeutic genes or transgenes are cloned under the control of a specific promoter, in particular a tumor-specific or tissue-specific promoter. It is also within the scope of such a virus that also the E4 region is functionally inactive, preferably is deleted. The transgenes described herein may also be cloned into the E4 region, whereby this may occur in addition or alternative to the cloning of a transgene into the E3 region and, respectively, the E3 region remains partially or completely intact. Transgenes as used herein, may be viral

genes, preferably adenoviral genes, which are preferably not present in the genome and which are not present at the site of the genome of the wildtype, respectively, where they are present in the particular virus now, or therapeutic genes.

Therapeutic genes may be prodrug genes, genes for cytokines, apoptosis-inducing genes, tumor suppressor genes, genes for metalloproteinase inhibitors and/or angiogenesis inhibitors. Additionally, siRNA, aptamers, antisense and ribozymes may be expressed which are directed against cancer-relevant target molecules. Preferably, the single or the multiple target molecules is/are selected from the group comprising resistance relevant factors, anti-apoptosis factors, oncogenes, angiogenesis factors, DNA synthesis enzymes, DNA repair enzymes, growth factors and their receptors, transcription factors, metalloproteinases, in particular matrix metalloproteinases and plasminogen activator of the urokinase type. Preferred embodiments thereof have already been disclosed herein.

Possible prodrug genes, which may be used in preferred embodiments, are, for example, *cytosine deaminase, thymidine kinase, carboxypeptidase, uracil phosphoribosyl transferase; purine nucleoside phosphorylase (PNP);* Kirn et al, Trends in Molecular Medicine, Volume 8, No.4 (Suppl), 2002; Wybranietz W.A. et al., Gene Therapy, 8, 1654-1664, 2001; Niculescu-Duvaz et al., Curr. Opin. Mol. Therapy, 1, 480.486, 1999; Koyama et al., Cancer Gene Therapy, 7, 1015-1022, 2000; Rogers et al., Human Gene Therapy, 7, 2235-2245, 1996; Lockett et al., Clinical Cancer Res., 3, 2075-2080, 1997; Vijayakrishna et al., J. Pharmacol. And Exp. Therapeutics, 304, 1280-1284, 2003.

Possible cytokines which may be used in preferred embodiments, are, for example, *GM-CSF, TNF-alpha, Il-12, Il-2, Il-6, CSF, interferon-gamma*; Gene Therapy, Advances in Pharmacology, Volume 40, Editor: J. Thomas August, Academic Press; Zhang und Degroot, Endocrinology, 144, 1393-1398, 2003; Descamps et al., J. Mol. Med., 74, 183-189, 1996; Majumdar et al., Cancer Gene Therapy, 7, 1086-1099, 2000.

Possible apoptosis inducing genes as may be used in preferred embodiments, are, for example, decorin: Tralhao et al., FASEB J, 17, 464-466, 2003; retinoblastoma 94: Zhang et al., Cancer Res.,63, 760-765, 2003; Bax and Bad: Zhang et al, Hum. Gene Ther., 20, 2051-2064, 2002; apoptin: Noteborn and Pietersen, Adv. Exp. Med. Biol., 465, 153-161, 2000); ADP: Toth et al., Cancer Gene Therapy, 10, 193-200, 2003; bcl-xs: Sumantran et al., Cancer

Res, 55, 2507-2512, 1995; E4orf4: Braithwaite and Russell, Apoptosis, 6, 359-370, 2001; FasL, Apo-1 and Trail: Boehringer Manheim, Guide to Apoptotic Pathways, Arai et al., PNAC, 94, 13862-13867, 1997; Bims; Yamaguchi et al., Gene Therapy, 10, 375-385, 2003; GNR163: Oncology News, 17 Juni, 2000.

Possible tumor suppressor genes as may be used in preferred embodiments, are, for example, *E1A, p53, p16, p21, p27, MDA-7.* Opalka et al., Cell Tissues Organs, 172, 126-132, 2002 , Ji et al., Cancer Res., 59, 3333-3339, 1999, Su et al., Oncogene, 22, 1164-1180, 2003.

Possible angiogenesis inhibitors as may be used in preferred embodiments are, for example, *endostatin, angiostatin*: Hajitou et al., FASEB J., 16, 1802-1804, 2002, and antibodies against VEGF (Ferrara, N., Semin Oncol 2002 Dec; 29 (6 Suppl 16): 10-4.

Possible metalloproteinase inhibitors as may be used in preferred embodiments are, for example, *Timp-3*, Ahonen et al., Mol Therapy, 5, 705-715, 2002; *PAI-1*; Soff et al., J. Clin. Invest., 96, 2593-2600, 1995; *Timp-1*, Brandt K. Curr. Gene Therapy, 2, 255-271, 2002.

siRNA (short interfering RNA) as used in connection with the present invention, consists of two, preferably two separate RNA strands, which hybridise with each other due to base complementarity, i. e. are essentially base paired and preferably have a length of up to 50 nucleotides, preferably between 18 and 30 nucleotides, more preferably less than 25 nucleotides and most preferably 21, 22 or 23 nucleotides, whereby these figures refer to the single strand of the siRNA, in particular to the length of the stretch of the single strand which hybridises with one, and more particularly with the second single strand and is base paired therewith, respectively. siRNA specifically induces or mediates the degradation of mRNA. The specificity required theretofor is provided by the sequence of the siRNA and thus its binding site. The target sequence to be degraded is essentially complementary to the first or to the second one of the siRNA forming strands. Although the exact mode of action is still unclear, it is assumed that siRNA represents a biological strategy for cells to inhibit certain alleles during development and to protect itself from viruses. siRNA mediated RNA interference is used for the specific suppression or even complete knock-out of the expression of a protein by introducing a gene specific double-stranded RNA. For higher organisms an siRNA having a length from 19 to 23 nucleotides, is thus particularly preferred as it does not result in activation of an inspecific defense reaction, the so-called interleukin response.

Immediate transfection of double-stranded RNA consisting of 21 nucleotides having symmetric 2-nt long overhangs at the 3' end was able to mediate RNA interference in mammal cells and was highly efficient compared to other technologies such as ribozymes and antisense molecules (Elbashir, S. Harborth J. Lendeckel W. Yalvcin, A. Weber K Tuschl T: Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 2001, 411: 494-498). Only very few siRNA molecules were necessary so as to inhibit the expression of the target gene. In order to avoid the limitations of exogenously administered siRNA which particularly resides in the transient nature of the interference phenomenon and the specific delivery of the siRNA molecules, the prior art uses vectors which allow for an endogenous siRNA expression. For example, oligonucleotides having a length of 64 nucleotides are provided which contain the 19 nucleotide long target sequence, both in sense as well as in antisense orientation, separated through a, for example, 9 nucleotide long spacer sequence which was introduced into the vector. The resulting transcript folded into a hairpin structure having a stem structure of, for example, 19 base pairs. The loop is rapidly degraded in the cell so that a functional siRNA is generated (Brummelkamp et al., Science, 296, 550-553, 2002).

The activity of pRb and E2F, respectively, is regulated through phosphorylation. The hypophosphorylated form of pRb is predominantly present in the G1 and M phase. In contrast, the hyperphosphorylated form of pRb is present in the S and G2 phase. E2F is released from the complex consisting of E2F and hypophosphorylated pRb by phosphorylation of pRb. The release of E2F from the complex consisting of E2F and hypophosphorylated pRb results in the transcription of E2F dependent genes. The E1A protein does not only bind to the hypophosphorylated form of pRb, whereby the binding of E1A to pRb happens mostly through the CR2 region of the E1A protein. Additionally, it also binds to the CR1 region, although with lower affinity (Ben-Israel and Kleiberger, Frontiers in Bioscience, 7, 1369-1395, 2002; Helt and Galloway, Carcinogenesis, 24, 159-169, 2003).

The nucleic acid coding for YB-1 which, in an embodiment of the adenoviruses to be used in accordance with the present invention, is part of the adenoviruses, may also comprise a nucleic acid sequence mediating the transport of YB-1 into the nucleus. The nucleic acids, adenoviruses and adenoviral systems in accordance with the invention as well as the adenoviruses known in the prior art such as, for example, Onyx-015, AdΔ24, dl922-947, E1Ad/01/07, CB016, dl 520 and the adenoviruses described in patent EP 0 931 830, can be

used as such or in combination with these nucleic acids in accordance with the invention in connection therewith as adenoviruses and adenoviral systems and thus as the corresponding nucleic acids. Suitable nucleic acid sequences which mediate nucleus transport, are known to the ones skilled in the art and, for example, described in (Whittaker, G.R. et al., Virology, 246, 1-23, 1998; Friedberg, E.C., TIBS 17, 347, 1992; Jans, D.A. et al., Bioessays 2000 Jun; 22(6): 532-44; Yoneda, Y., J. Biocehm. (Tokyo) 1997 May; 121(5): 811-7; Boulikas, T., Crit. Rev. Eukaryot. Gene Expr. 1993; 3(3): 193-227; Lyons RH, Mol. Cell Biol., 7, 2451-2456, 1987). In connection with the nucleus transport mediating nucleic acid sequences, different principles can be used. One such principle may, for example, be that YB-1 is formed as a fusion protein together with a signal peptide and is introduced into the nucleus and that the replication of the adenoviruses according to the present invention thus occurs.

A further principle which may be realised in the design of the adenoviruses used in accordance with the invention, is that YB-1 can be provided with a transporter sequence which, preferably starting from synthesis in the cytoplasma, introduces YB-1 into the cell nucleus or which translocates YB-1 into the cell nucleus, and promotes viral replication there. An example for a particularly effective nucleic acid sequence mediating nucleus transport is the TAT sequence of HIV which is, among other suitable nucleic acid sequences of that type described in Efthymiadis, A., Briggs, LJ, Jans, DA., JBC 273, 1623-1628, 1998. It is within the present invention that the adenoviruses which are used in accordance with the present invention, comprise nucleic acid sequences which code for peptides coding for nuclear transportation.

It is within the present invention that YB-1 is present in its full length, particularly in a form which corresponds to the wildtype of YB-1. It is within the present invention that YB-1 is used or present as a derivative, such as, e. g. in shortened or truncated form. A YB-1 derivative as used or present within the present invention, is a YB-1 which is capable of binding to the E2-late promoter and thus activates gene expression of the adenoviral E2 region. Such derivatives particularly comprise the YB-1 derivatives disclosed herein. Further derivatives may be generated by deletion of single or several amino acids at the N-terminus, at the C-terminus or within the amino acid sequence.

With regard to the previously mentioned various further expressed genes and gene products coded by the adenoviruses, it is also possible that these are coded in any combination and expressed in any combination, respectively.

The use of the adenovirus as disclosed herein as medicaments and in particular in case of systemic administration can be improved by a suitable targeting of the adenoviruses. The infection of tumor cells by adenoviruses depends to a certain extent on the presence of the coxackievirus-adenovirus receptor CAR and certain integrins. If these are strongly expressed in cells, in particular tumor cells, an infection is possible already at very low titers (pfu/cell). Various strategies have been practiced so far in order to obtain a so-called re-targeting of the recombinant adenoviruses, for example, by insertion of heterologuous sequences into the fiber knob region, use of bi-specific antibodies, coating of the adenoviruses with polymers, introduction of ligands into the Ad fiber, the substitution of the serotype 5 knob and fiber shaft and knop with the serotype 3 knop and Ad 35 fiber shaft and knob, respectively, and modification of the penton base (Nicklin S. A. et al., Molecular Therapy 2001, 4, 534-542; Magnusson, M. K. et. al., J. of Virology 2001, 75, 7280-7289; Barnett B. G. et al., Biochimica et Biophysica Acta 2002, 1575, 1-14). The realization of such further embodiments and features, respectively, in connection with the adenoviruses of the present invention and the adenoviruses used in accordance with the present invention in the various aspects of the present invention is within scope of the present invention.

The present inventor has furthermore surprisingly found that the efficacy of the viruses described herein and in particular the viruses used in accordance with the present invention can be increased by using them in combination with at least two agents whereby each of the at least two agents is individually and independently selected from the group comprising cytostatics.

As used herein in a preferred embodiment, cytostatics are in particular chemical or biological compounds which, during or after the administration to a cell or an organism containing a or such cell, result in the cell no longer growing and/or no longer dividing or slowing down cell division and/or cell growth. Cytostatics also comprise compounds which turn into a cytostatic in the afore-described sense only in the cell or in an organism containing such cell. Insofar, the term cytostatics also comprises pre-cytostatics.

Cytostatics are grouped according to their mode of action. The following groups are distinguished which, in principle, can all be used within the present invention:

- Alkylating agents, i. e. chemical compounds which cause their cytotoxic effect by alkylating phosphate, amino, sulphydryl, carboxy and hydroxy groups of the nucleic acid as well as proteins. Such compounds are often cancerogenic themselves. Typical examples of this group of cytostatics are cis-platin and platin derivatives, cyclophosphamide, dacarbazine, mitomycin, procarbazine.

- Antimetabolites, i. e. compounds which, due to their structural similarity or ability for binding block a metabolic process or affect the same. Within the group of antimetabolites it is distinguished between structurally similar antimetabolites, structure changing antimetabolites and the indirectly acting antimetabolites. The structurally similar antimetabolites compete due to chemical similarity with the metabolite without exerting the function thereof. Structure changing antimetabolites bind to the metabolites which impedes its function or resorption or chemically modifies the metabolite. Indirectly acting antimetabolites interfere with the function of the metabolite, for example by the binding of ions. Typical examples of this group are folic acid antagonists such as methotrexate, pyrimidine analogues such as fluorouracil, purine analogues such as azathioprine and mercaptopurine.

- Mitosis inhibitors, i. e. compounds which inhibit cell division. Within the group of mitosis inhibitors it is distinguished between cell division toxins, spindle toxins and chromosome toxins. Typical examples of this group are taxanes and vinca alkaloids. The taxanes in turn can be divided into the two major groups of taxoles and taxoters, whereby a particularly preferred taxole is paclitaxel, and a particularly preferred taxoter is docetaxel.

- Antibiotics having an inhibitory effect on the DNA-dependent RNA polymerase. Typical examples are the anthracyclines, such as, e. g., bleomycin, daunorubicin, doxorubicin and mitomycin.

- Topoisomerase inhibitors, in particular topoisomerase I inhibitors. Topoisomerase inhibitors are chemical compounds which determine the tertiary structure of the DNA by catalysing the change of the DNA twist number in a three stage process. Essentially, two forms of topoisomerases are distinguished. Topoisomerases of type I cleave only a DNA

strand and are ATP-independent, whereas topoisomerase of type II cleave both strands of a DNA, whereby they are ATP-dependent. Typical examples for topoisomerase I inhibitors are irinotecan and topotecan, and for topoisomersae II inhibitors etoposid and daunorubicin.

Within the present invention at least one and preferably two agents are selected from the aforementioned group. It is, however, also within the invention that in particular also three, four or five different agents are selected. The following comments are made for the embodiment of the present invention where only two agents are used together with the virus. These considerations are basically also applicable to embodiments where more than two agents are used.

Preferably the agents differ from each other such that they address or target different target molecules or are described in the literature as targeting different molecules. It is within the present invention that the agent also comprises two or more different agents which bind to the same target molecule. It is also within the present invention that one agent binds to a first site of the target molecule, whereas the second agent binds to a second site of the target molecule.

It is also within the present invention that at least two of the agents are active using different modes of action. Active means in a preferred embodiment that the cell growth and/or cell division inhibiting or retarding effect of the chemical compound is mediated through a different mode of action. In a particularly preferred embodiment the term active means that the replication efficiency of a virus, in particular the virus in accordance with the present invention, of the viruses described herein and of the viruses to be used in accordance with the present invention, is increased compared to a scenario where one and/or both of the agents are not used. As a measure for the efficiency of viral replication preferably the number of viruses required for cell lysis is used, preferably expressed as pfu/cell.

In a particularly preferred embodiment at least one of the at least two agents is one which increases the infectability of the cell in which the replication of the virus is to occur, preferably is to occur in a selective manner, preferably with the virus described herein and/or the virus to be used in accordance with the present invention. This can, e. g., be performed by increasing the uptake of the virus by the cell. The uptake of the virus, in particular of adenovirus, is, for example, mediated by the coxsackievirus-adenovirus receptor (CAR) (Mizuguchi und Hayakawa, GENE 285, 69-77, 2002). An increased expression of CAR is, for

example, caused by trichostatin A (Vigushin et al., Clinical Cancer Research, 7, 971-976, 2001).

In a further embodiment one of the at least two agents is one which increases the availability of a component within the cell, whereby the component is one which increases the replication of the virus, preferably the virus described herein and/or the virus to be used in accordance with the present invention.

In a further embodiment one of the at least two agents is one which mediates the transport of YB-1 into the nucleus. Such an agent can be selected from the group comprising topoisomerase inhibitors, alkylating agents, antimetabolites and mitosis inhibitors. Preferred topoisomerase inhibitors are camptothecin, irinotecan, etoposide and their respective analogues. Preferred mitosis inhibitors are daunorubicin, doxorubicin, paclitaxel and docetaxel. Preferred alkylating agents are cis-platin and their analogues. Preferred antimetabolites are fluorouracil and methotrexat.

In a particularly preferred embodiment one of the at least two agents is one which increases the infectability of the cell, in particular the expression of CAR, and the second of the at least two agents is one which increases the transport of YB-1 into the nucleus, whereby preferably as chemical compound a compound is used which exhibits the respective required characteristic as preferably described above.

In a further embodiment the one of the at least two agents is a histone deacylase inhibitor. A preferred histone deacylase inhibitor is one which is selected from the group comprising trichostatin A, FR901228, MS-27-275, NVP-LAQ824 and PXD101. Trichostatin A is, for example, described in Vigushin et al., Clinical Cancer Research, 7, 971-976, 2001; FR901228 is, for example, described in Kitazono et al., Cancer Res., 61, 6328-6330, 2001; MS-27-275 is described in Jaboin et al., Cancer Res., 62, 6108-6115, 2002; PXD101 is described in Plumb et al., Mol. Cancer Ther., 8, 721-728, 2003; NVP-LAQ824 is described in Atadja et al., Cancer Res., 64, 689-695, 2004.

In a still further embodiment the one of the at least two agents is a topoisomerase inhibitor, preferably a topoisomerase I inhibitor. A preferred topoisomerse inhibitor is one which is selected from the group comprising camptothecin, irinotecan, topotecan, SN-38, 9-

aminocamptothecin, 9-nitrocamptothecin, DX-895If and daunorubicin. Irinotecan and SN-38 are, for example, described in Gilbert et al., Clinical Cancer Res., 9, 2940-2949, 2003; DX-895IF is described in van Hattum et al., British Journal of Cancer, 87, 665-672, 2002; camptothecin is described in Avemann et al., Mol. Cell. Biol., 8, 3026-3034, 1988; 9-aminocamptothecin, 9-nitrocamptothecin are described in Rajendra et al., Cancer Res., 63, 3228-3233, 2003; daunorubicin is described in M. Binaschi et al., Mol. Pharmacol., 51, 1053-1059.

In a particularly preferred embodiment the one of the at least two agents is a histone deacylase inhibitor and the other one of the at least two agents is a topoisomerse inhibitor.

In an embodiment the means according to the present invention and/or the means prepared in accordance with the present invention contains the virus separate from one or several of the at least two agents which are combined with the virus in accordance with the present invention. It is preferred that the virus is separate from any agent which is combined with the virus. Preferably the separation is a spatial separation. The spatial separation can be such that the virus is present in a different package than the agent. Preferably the package is a single dose unit, i. e. the virus and the agent are packed as single dosages or doses. The single dose units may in turn be combined to form a package. However, it is also within the present invention that the single dosages of the virus are combined with one or several single dosages of one or several of the agents or are packed therewith.

The kind of package depends on the way of administration as known to the one skilled in the art. Preferably the virus will be present in a lyophilized form or in a suitable liquid phase. Preferably, the agents will be present in solid form, e. g. as tablets or capsules, however, are not limited thereto. Alternatively, also the agents can be present in liquid form.

It is within the present invention that the virus is systemically or locally administered. It is also within the present invention that the agents combined with the virus are systemically or locally administered individually and independently from each other or together. Other modes of administration are known to the ones skilled in the art.

It is also within the present invention that the virus and the agents combined with it, are administered in a chronologically separate manner or at the same time. In connection with a

chronologically separate manner it is preferred that the agents are administered prior to the administration of the virus. How long the agent is administered prior to the virus depends on the kind of the agent used and is obvious for the one skilled in the art from the mode of action of the agent used. Also the administration of the at least two agents can occur at the same or at different points in time. In connection with a chronologically different administration the points of time again result from the modes of action underlying the agents and can, based thereon, be determined by the ones skilled in the art.

The above considerations, given in connection with the medicaments according to the present invention which are also disclosed and referred to herein as pharmaceutical compositions, are roughly also applicable to any composition, including compositions as used for the replication of viruses, preferably for the *in vitro* replication of viruses in accordance with the present invention. The above considerations are also applicable to the kit in accordance with the present invention and the kit to be used in accordance with the present invention, respectively, which may apart from the viruses described herein and the viruses to be used in accordance with the invention, also comprise one agent or a combination of agents as described herein. Such kits comprise the virus and/or one or several agents in a form ready for use, and preferably instructions for use. Furthermore, the above embodiments apply also to the nucleic acids as disclosed herein, and the nucleic acids used in accordance with the present invention, and the replication systems in accordance with the present invention and the nucleic acids coding therefor, and, respectively, the replication systems used in accordance with the present invention and the nucleic acids coding therefor used in accordance with the present invention.

The terms adenovirus and adenoviral system shall have essentially the same meaning within the present invention. Adenovirus shall particularly refer to the complete viral particle comprising the capsid and the nucleic acid. The term adenoviral system particularly focuses on the fact that the nucleic acid shall be changed compared to the wildtype. Preferably such changes comprise changes in the structure of the genome of the adenovirus as may arise from deleting and/or adding and/or mutating promoters, regulatory sequences and/or coding sequences such as open reading frames. Additionally, the term adenoviral system is preferably used in connection with a vector, which is, for example, used in gene therapy.

The previously provided comments, including any use as well as design of the adenoviruses and adenoviral systems, respectively, apply also to the coding nucleic acids and vice versa.

In connection with the present invention it is possible that the adenoviruses to be used in accordance with the present invention and the nucleic acids coding therefor, respectively, may be any respective adenoviral nucleic acid which results in a replication event as such or in combination with further nucleic acid sequences. It is possible, as explained herein, that by means of helper virus the sequences and/or gene products required for replication are provided. To the extent it is referred to coding nucleic acid sequences and to the extent that such nucleic acid sequences are known, it is within the invention that not only the identical sequences used but also sequences derived therefrom. The term derived sequences shall in particular refer herein to sequences which still result in a gene product, either a nucleic acid or a polypeptide, that exhibits a function which corresponds to one or the function of a non-derived sequence. This can be determined by simple routine tests known to the one skilled in the art. An example for such derived nucleic acid sequences are those nucleic acid sequences which code for the same gene product, in particular for the same amino acid sequence, however, have a deviating sequence of bases due to the degeneracy of the genetic code.

In a preferred embodiment, with regard to the adenoviruses according to the present invention and the adenoviral replication system according to the present invention and the use of them according to the present invention, respectively, the adenoviral nucleic acid is deficient for the expression of the oncogene protein, particularly of the E1A protein, which means that it is either not coding for the 12S E1A protein or for the 13S E1A protein, or it is neither coding for the 12S E1A protein nor the 13S E1A protein, or is modified, as defined herein, and that the adenoviral replication system further comprises a nucleic acid of a helper virus, whereby the nucleic acid of the helper virus comprises a nucleic acid sequence which codes for the oncogene protein, in particular for the E1A protein, which has the following characteristics and imparts the following characteristics to the adenovirus, respectively, namely that it preferably is not replicating in YB-1 nucleus-negative cells but in cells which are independent from the cell cycle YB-1 nucleus-positive, transactivating at least one viral gene, in particular E1B55kDa, E4orf6, E4orf3 and/or E3ADP, in YB-1 nucleus-positive cells, and/or does not translocate cellular YB-1 into the nucleus. It is within the present invention that the transgenes described herein are coded individually or together by the helper virus and/or expressed therefrom.

In an embodiment of such an adenoviral replication system according to the present invention the adenoviral nucleic acid and/or the nucleic acid of the helper virus are furthermore present as vectors which are capable of replicating.

It is within the present invention that the coding nucleic acid(s) coding for the adenoviruses which are used according to the present invention, is/are present in a vector, preferably in an expression vector and this expression vector is used in accordance with the present invention.

In a further aspect the present invention is also related to a vector group comprising at least two vectors, whereby the vector group comprises altogether an adenoviral replication system as described herein, and the vector group is used in accordance with the present invention. It is intended that each of the components of the adenoviral replication system is arranged on an individual vector, preferably an expression vector.

Finally, the present invention is related in a further aspect to the use of a cell for the same purposes as described herein for the adenoviruses, whereby the cell comprises one or several nucleic acids which code for the adenoviruses described herein to be used in accordance with the invention and/or a respective adenoviral replication system and/or a respective vector and/or a vector group according to the present invention.

The previously described constructs of adenoviruses and in particular their nucleic acids and the nucleic acids coding therefor, may also be introduced into a cell in parts, particularly into a tumor cell, whereupon due to the presence of the various individual components they may act together such as if the individual components originated from a single nucleic acid and a single or several adenoviruses, respectively.

The nucleic acids coding for adenoviruses, adenoviral systems or parts thereof, which are used in accordance with the invention, may be present as vectors. Preferably, they are present as viral vectors. In case of nucleic acids comprising adenoviral nucleic acids the virus particle is preferably the vector. However, it is also within the invention that said nucleic acids are present in a plasmid vector. In any case the vector comprises elements which provide for the propagation of the inserted nucleic acid, i. e. replication and optionally expression of the inserted nucleic acid, and control of them, respectively. Suitable vectors, in particular expression vectors, and corresponding elements are known to the ones skilled in the art and,

for example, described in Grunhaus, A., Horwitz, M.S., 1994, Adenoviruses as cloning vectors. In Rice, C., edit., Seminars in Virology, London: Saunders Scientific Publications.

The aspect of the invention that is related to the vector group, accounts for the previously described embodiment, that the various elements of the nucleic acid are not necessarily contained on one vector only. Accordingly, a vector group comprises at least two vectors. Otherwise, what has been said in relation to the vectors is also applicable to the vectors and the vector group, respectively.

The adenoviruses which are used in accordance with the invention are characterised by various nucleic acids and gene products, respectively, disclosed herein, and may otherwise comprise all those elements known to the one skilled in the art, as is also the case for adenoviruses of the wildtype (Shenk, T.: Adenoviridae: The virus and their replication. Fields Virology, 3rd edition, edit. Fields, B.N., Knipe, D.M., Howley, P.M. et al., Lippincott-Raven Publishers, Philadelphia, 1996, chapter 67).

The replication of adenoviruses is a very complex procedure and usually makes use of the human transcription factor E2F. During viral infection, first, the "early genes" E1, E2, E3 and E4 are expressed. The group of the "late genes" is responsible for the synthesis of the viral structural proteins. For the activation of both the early as well as the late genes, the E1 region consisting of the two transcriptional units E1A and E1B, which code for different E1A and E1B proteins, are critical as the transcription of the E2, E3 and E4 is induced by them (Nevins, J. R., Cell 26, 213-220, 1981). Additionally, the E1A proteins can induce DNA synthesis in resting cells and thus initiate the entry into the S phase (c. f. Boulanger and Blair, 1991). Additionally, they interact with the tumor suppressors of the Rb class (Whyte, P. et al., Nature 334, 124-127, 1988). In doing so, the cellular transcription factor E2F is released. The E2F factors may subsequently bind to corresponding promoter regions of both cellular as well as viral genes (in particular to the adenoviral E2 early promoter) and thus initiate transcription and replication (Nevins, J. R., Science 258, 424-429, 1992).

The gene products of the E2 region are especially needed for the initiation and performance, respectively, of the replication, as they code for three essential proteins. The transcription of the E2 proteins is controlled by two promoters, the "E2-early E2F-dependent" promoter which is also referred to herein as E2-early promoter or early E2 promoter, and the "E2-late"

promoter (Swaminathan and Thimmapaya, The Molecular Repertoire of Adenoviruses III: Current Topics in Microbiology and Immunology,Vol 199, 177-194, Springer Verlag 1995). Additionally, the products of the E4 region together with the E1A and E1B-55kDa protein play an important role for the activity of E2F and the stability of p53, respectively. For example, the promoter is even more activated by a direct interaction of the E4orf6/7 protein coded by the E4 region, with the heterodimer consisting of E2F and DP1 (Swaminathan and Thimmapaya, JBC 258, 736-746, 1996). Furthermore, p53 is inactivated by the complex consisting of E1B-55kDa and E4orf6 (Steegenga, W. T. et al., Oncogene 16, 349-357, 1998), in order to successfully complete a lytic infectious cycle. Additionally, the E1B-55kDa protein exhibits a further important function insofar as it promotes by interacting with the E4orf6 protein the export of viral RNA from the nucleus, whereas the proprietary RNAs of the cell are retained in the nucleus (Bridge and Ketner, Virology 174, 345-353, 1990). A further important discovery is that the protein complex consisting of E1B-55kDa/E4orf6 is localised in the so-called "viral inclusion bodies". It is assumed that these structures are the sites of replication and transcription (Ornelles and Shenk, J. Virology 65, 424-429, 1991).

A further region which is important for replication and in particular for the release of adenoviruses, is the E3 region. The E3 region comprises more particularly the genetic information for a variety of comparatively small proteins which are not essential for the adenoviral infectious cycle in vitro, i. e. are not essential in cell culture. However, they play an important role for the survival of the virus during an acute and/or latent infection in vivo, as they have, among others, immune regulatory and apoptotic function(s) (Marshall S. Horwitz, Virololgie, 279, 1-8, 2001; Russell, supra). It could be shown that a protein having a size of about 11.6 kDa induces cell death. The protein was, due to its function, referred to as ADP – for the english term adenovirus death protein – (Tollefson, J. Virology, 70, 2296-2306, 1996). The protein is predominantly formed in the late phase of the infectious cycle. Furthermore, overexpression of the protein results in a better lysis of the infected cells (Doronin et al., J. Virology, 74, 6147-6155, 2000).

Furthermore, it is known to the present inventor that E1A deleted viruses, i. e. in particular viruses which do not have a 12S E1A protein and which also do not express a 13S E1A protein, can very efficiently replicate at higher MOIs (Nevins J. R., Cell 26, 213-220, 1981), which, however, cannot be realised in any clinic application. This phenomenon is referred to as "E1A-like activity" in literature. It is was also known that from the 5 proteins coded by

E1A, two proteins, namely the 12S and 13S protein, control and induce, respectively, expression of the other adenoviral genes (Nevins, J. R., Cell 26, 213-220, 1981; Boulanger, P. und Blair, E.; Biochem. J. 275, 281-299, 1991). In connection therewith it was shown that the transactivating function is predominantly provided by the CR3 region of the 13S protein (Wong HK und Ziff EB., J Virol., 68, 4910-20, 1994). Adenoviruses which have specific deletions in the CR1 and/or CR2 region and/or CR3 region of the 13S protein, are mostly replication-deficient, however, are still transactivating in some cell lines the viral genes and promoters, respectively, in particular the E2 region (Wong HK, Ziff EB., J Virol. 68, 4910-20, 1994; Mymryk, J. S. and Bayley, S. T., Virus Research 33, 89-97, 1994).

After infection of a cell, typically a tumor cell, using a wildtype adenovirus, YB-1 is induced into the nucleus which is mediated by E1A, E1B-55K and E4orf6, and is co-localised with E1B-55K in the nucleus in the viral inclusion bodies, which allows for an efficient replication of the virus in the cell nucleus both in vitro and in vivo. In connection therewith, it has already been found earlier that E4orf6 binds to E1B-55K (Weigel, S. and Dobbelstein , M. J. Virology, 74, 764-772, 2000; Keith N. Leppard, Seminars in Virology, 8, 301-307, 1998) and thus mediates the transport and distribution, respectively, of E1B-55K into the nucleus, which provides for an optimum virus production and adenoviral replication, respectively. An efficient replication of the virus in accordance with the present invention is possible due to the interaction of E1A, E1B-55K and YB-1, and by the complex consisting of E1B-55K/E4orf6 with YB-1, respectively, and the co-localisation of YB-1 and E1B-55K in the nucleus in the so-called viral inclusion bodies and thus the use of the viruses described herein for replication in cells which are YB-1 nucleus-positive and for the manufacture of a medicament for the treatment of diseases, whereby YB-1 nucleus-positive cells are involved. The replication being thus possible with this cellular background, results in lysis of the cell, release of the virus and infection and lysis of adjacent cells, so that in case of an infection of a tumor cell and a tumor, respectively, finally lysis of the tumor, i. e. oncolysis, occurs.

YB-1 belongs to the group of highly conserved factors which bind to an inverted CAAT sequence, the so-called Y-box. They may be active in a regulatory manner both at the level of transcription as well as translation (Wolffe, A. P. *Trends in* Cell Biology 8, 318-323, 1998). A growing number of Y-box dependant regulatory pathways is found in the activation but also in the inhibition of growth and apoptosis associated genes (Swamynathan, S. K. et al.,FASEB J. 12, 515-522, 1998). Accordingly, YB-1 directly interacts with p53 (Okamoto, T. et al.,

Oncogene 19, 6194-6202, 2000), plays an important role in the gene expression of Fas (Lasham, A. et al., Gene 252, 1-13, 2000), MDR and MRP gene expression (Stein, U. et al., JBC 276, 28562-69, 2001; Bargou, R. C. et al., Nature Medicine 3, 447-450, 1997) and in the activation of topoisomerases and metalloproteinases (Mertens. P. R. et al., JBC 272, 22905-22912, 1997; Shibao, K. et al., Int. J. Cancer 83, 732-737, 1999). Additionally, YB-1 is involved in the regulation of mRNA stability (Chen, C-Y. et al., Genes & Development 14, 1236-1248, 2000) and repair processes (Ohga, T. et al., Cancer Res. 56, 4224-4228, 1996).

The nuclear localisation of YB-1 in tumor cells results in E1A independent viral replication whereby in particular neither a 12S E1A protein nor a 13S E1A protein is present in an expressed form and used, respectively (Holm, P. S. et al. JBC 277, 10427-10434, 2002) and in case of overexpression of the protein YB-1 in multidrug resistance (multiple resistance). Additionally it is known that the adenoviral proteins such as e. g. E4orf6 and E1B-55K have a positive effect on viral replication (Goodrum, F. D. and Ornelles, D. A, J. Virology 73, 7474-7488, 1999), whereby a functional E1A protein is responsible for switching on the other viral gene products (such as E4orf6, E3ADP and E1B-55K) (Nevins J. R., Cell 26, 213-220, 1981). This, however, does not occur with the E1A-minus adenoviruses of the prior art in which the 13S E1A protein is not present. The nuclear localisation of YB-1 in multidrug resistant cells which have YB-1 in the nucleus, provides for replication and particle formation, respectively, of such E1A-minus viruses. In this case, however, the efficiency of viral replication and particle formation is reduced by several multiples compared to wildtype Ad5. A combination of YB-1 which is either already present in the nucleus of the tumor cell, or is induced into the tumor cell by external factors (e. g. application of cytostatics or irradiation or hyperthermia), i. e. is prompted to be present in the nucleus, particularly independent from the cell cycle, or is introduced as a transgene through a vector, with a system, preferably with an adenoviral system, which switches on adenoviral genes, but which does not allow for viral replication, has been surprisingly found to be a system which mediates a very efficient viral replication and particle formation through YB-1 and thus provides oncolysis. Suitable cytostatics are, among others, those which belong to the following groups: anthracyclines, such as daunomycin and adriamycin; alkylating agents, such as cyclophosphamide; alkaloids, such as etoposide; vin-alkaloids, such as vincristine and vinblastine; antimetabolites such as for example 5-fluorouracil and methrotrexate; platin derivatives, such as for example cis-platin; topoisomerase inhibitors, such as camphothecine; and taxanes, such as for example taxole. The adenoviruses disclosed herein, in particular the recombinant adenoviruses, which are only

capable of replicating in YB-1 nucleus-positive cells, are limited in their capability to transactivate the viral genes E1B-55K, E4orf6, E4orf3 and E3ADP, compared to the corresponding transactivating capabilities of wildtype adenoviruses, in particular wildtype Ad5. The present inventor has now surprisingly found that these limited transactivating capabilities may be compensated by the corresponding genes and in particular by E1B-55K and E4orf6 being expressed in combination with the nuclear localisation of YB-1. As shown in the examples herein, viral replication and particle formation, respectively, is increased under such conditions to a level which is nearly comparable to the replication and particle formation behaviour of wildtype adenoviruses.

It is intended that the medicament in connection with which or for the manufacture of which the adenoviruses described herein are used in accordance with the present invention, is usually applied systematically, although it is also within the present invention to apply or deliver such medicament locally. The application is done with the intention that particularly those cells are infected with the adenovirus and that particularly in these cells replication occurs, which are involved, preferably in a causal manner, in the formation of a condition, typically a disease, for the diagnosis and/or prevention and/or treatment of which the medicament according to the present invention is used.

Such a medicament is preferably for the treatment of tumor diseases. Among the tumor diseases, those are particularly preferred in which either YB-1 is already located in the nucleus due to the mechanism underlying the tumor disease, in particular the underlying pathological mechanism, or those where the presence of YB-1 in the nucleus is caused by exogenous measures, whereby the measures are suitable to transfer YB-1 into the nucleus, induce YB-1 there or to express YB-1 there. The term tumor or tumor disease as used herein shall comprise both malignant as well as benign tumors and respective diseases. It can be intended that the medicament comprises at least one further pharmaceutically active compound. The kind and the amount of such further pharmaceutically active compound will depend on the indication for which the medicament is to be used. In case the medicament is used for the treatment and/or prevention of tumor diseases, typically cytostatics, such as for example cis-platin and taxol, daunoblastin, daunorubicin, adriamycin (doxorubicin) and/or mitoxantron or others of the cytostatics or groups of cytostatics which are described herein, are used.

The medicament according to the present invention can be present in various formulations, preferably in a liquid form. Furthermore, the medicament will contain stabilisers, buffers, preservatives and such agents which are known to the one skilled in the art of pharmaceutical formulations.

The present inventor has surprisingly found that the use in accordance with the invention of the viruses described herein can be applied with a very high success rate to tumors which have YB-1 in the nucleus independent from the cell cycle. Normally, YB-1 is located in the cytoplasm, in particular also in the perinuclear plasma. During S-phase of the cell cycle, YB-1 can be found in the cell nucleus of both normal cells as well as tumor cells. This, however, is not sufficient to provide for viral oncolysis using thus modified adenoviruses. The comparatively little efficacy of such attenuated adenoviruses described in the prior art is ultimately based on their wrong application. In other words, such adenoviral systems can be used, particularly also with an increased efficacy, where the molecular biological prerequisites for viral oncolysis are given when the attenuated or modified viruses as described herein, are administered. In case of the described adenoviruses which are to be used in accordance with the invention as described herein, such as AdΔ24, dl922-947, E1Ad/01/07, CB016, dl520 and the recombinant adenoviruses described in European patent EP 0 931 830, the prerequisites are given in case of tumor diseases the cells of which show a nuclear localisation of YB-1 independent of the cell cycle. This form of nuclear localisation may be either caused by the kind of tumor itself or may be caused by the measures or agents in accordance with the invention which are described herein. The present invention thus defines a new group of tumors and tumor diseases, respectively, and thus also of patients, which can still be treated successfully with the viruses in accordance with the invention, particularly also with the attenuated or modified adenoviruses already described in the prior art.

A further group of patients which can be treated in accordance with the present invention using the adenoviruses, some of which are known and which can be used in accordance with the present invention, or using the adenoviruses which are described herein for the first time, in particular using such adenoviruses which have mutations and deletions, respectively, in the E1A protein which do not interfere with the binding of Rb/E2f, but which do not replicate in YB-1 nucleus-negative cells or which have and show, respectively, a strongly reduced replication as defined herein, and/or a deleted oncoprotein, particularly E1A, such as, for example, the viruses AdΔ24, dl922-947, E1Ad/01/07, CB106 and the adenoviruses described

in European patent EP 0 931 830, are those patients for which it is ensured that by applying or realising distinct conditions YB-1 is migrating into the nucleus or is induced there or is transported therein. The use of such adenoviruses in connection with this group of patients is based on the finding that the induction of viral replication is based on the nuclear localisation of YB-1 with subsequent binding of YB-1 to the E2-late promoter. Due to the findings disclosed herein adenoviruses such as AdΔ24, dl922-947, E1Ad/01/07, CB106 and/or the adenoviruses described in European patent EP 0 931 830 may also replicate in cells which are YB-1 nucleus-positive and/or in cells in which YB-1 is deregulated as defined in the present invention. Insofar, these adenoviruses can, according to the present invention, be used for the treatment of diseases and groups of patients, respectively, which/who comprise cells having these characteristics, particularly when these cells are involved in the formation of the respective disease to be treated. This is the basis for the success of AdΔ24, dl922-947, E1Ad/01/07, CB016 and the adenoviruses described in patent EP 0 931 830 for the treatment of such tumors, in accordance with the present invention, which have YB-1 in the nucleus independent of the cell cycle or in which YB-1 is deregulated in the sense of the present disclosure. A further group of patients which can be treated in accordance with the invention using the adenoviruses which are described herein as being usable in accordance with the invention, and using those viruses, in particular adenoviruses, which are described herein for the first time, are those which are YB-1 nucleus-positive and/or which are YB-1 nucleus-positive as a result of the treatments described in the following, whereby such treatment is preferably a medical treatment, and/or those patients which have undergone such treatment concomitantly with the administration of respective viruses. It is within the present invention that YB-1 nucleus-positive patients are patients which have YB-1 in the nucleus independent from the cell cycle in a number of cells forming a tumor. Among these treatments is the administration of cytostatics as described herein altogether and/or as used in a tumor therapy. Additionally, radiation, preferably radiation as used in a tumor therapy, belongs to this group of treatments. Radiation means in particular radiation with high energy radiation, preferably radioactive radiation, preferably as used in tumor therapy. Hyperthermia and the application of hyperthermia, preferably hyperthermia as used in tumor therapy, are further treatments. In a particularly preferred embodiment hyperthermia is applied locally. Finally, hormon treatment, particularly hormon treatment as used in tumor therapy, is a further treatment. In connection with such hormon treatment anti-estrogens and/or anti-androgens are used. Anti-estrogens such as tamoxifene, particularly in the therapy of breast cancer, and anti-androgens

such as for example flutamide or cyproterone acetate, are used in the therapy of prostate cancer.

It is within the present invention that some of the cells forming the tumor comprise YB-1 either inherently or after induction and active introduction into the nucleus, respectively, or comprise deregulated YB-1 in the sense of the present disclosure. Preferably, about 5 % or any percentage above, i. e. 6 %, 7 %, 8 % etc. of the tumor forming cells are such YB-1 nucleus-positive cells or cells in which YB-1 is present in a deregulated manner. Nuclear localisation of YB-1 can be induced by stress applied from outside and by locally applied stress, respectively. This induction can, for example, occur by means of radiation, in particular UV radiation, application of cytostatics, as, among others, already disclosed herein, and hyperthermia. In connection with hyperthermia it is essential that it can be realised now in a very specific manner, more particularly in a locally very specific manner, and may thus also provide for a specific nuclear localisation of YB-1 in the cell nucleus and, because of this, provide the prerequisites for a replication of the adenovirus and thus for cell and tumor lysis, which is preferably locally limited (Stein U, Jurchott K, Walther W, Bergmann S, Schlag PM, Royer HD. J Biol Chem. 2001,276(30):28562-9; Hu Z, Jin S, Scotto KW. J Biol Chem. 2000 Jan 28; 275(4):2979-85; Ohga T, Uchiumi T, Makino Y, Koike K, Wada M, Kuwano M, Kohno K. J Biol Chem. 1998, 273(11):5997-6000).

The medicament according to the present invention could thus also be administered to patients and groups of patients, or may be intended for them, where through appropriate pretreatment or concomitant treatment a transport of YB-1 is affected, preferably in the respective tumor cells.

Based on this technical teaching it is for the person of the art within his skills to perform suitable modifications particularly on E1A which, for example, may comprise deletions or point mutations in order to thus generate various embodiments of the adenoviruses, which may be used in connection with the use in accordance with the invention.

As has already been explained above, the adenoviruses which are used in accordance with the present invention, are capable of replicating in such cells and cellular systems, respectively, which have YB-1 in the nucleus. For answering the question whether the adenoviruses used in accordance with the present invention are able to replicate and are thus able to lyse the tumor,

the status of the cells with regard to the presence or absence of Rb, i. e. the retinoblastome tumor suppressor product, is irrelevant. Additionally, it is in connection with the use in accordance with the invention of said adenoviruses, not essential to take into consideration the p53 status of the infected cells, the cells to be infected or the cells to be treated, as by using the adenoviral systems as disclosed herein in connection with YB-1 nucleus-positive cells, i. e. cells which have YB-1 in the nucleus irrespective of the cell cycle, this p53 status as well as the Rb status do not have an impact on the performance of the technical teaching disclosed herein.

The oncogene and oncogene protein, respectively, in particular E1A, can be either under the control of the proprietor natural adenoviral promoters and/or be controlled by means of a tumor or tissue specific promoter. Suitable non-adenoviral promoters can be selected from the group comprising cytomegalovirus promoter, RSV (Rous sarcoma virus) promoter, adenovirus-based promoter Va I and the non-viral YB-1 promoter (Makino Y. et al., Nucleic Acids Res. 1996, 15, 1873-1878). Further promoters which can be used in connection with each and any aspect of the invention disclosed herein, comprise the telomerase promoter, the alpha-fetoprotein (AFP) promoter, the caecinoembryonic antigen promoter (CEA) (Cao, G., Kuriyama, S., Gao, J., Mitoro, A., Cui, L., Nakatani, T., Zhang, X., Kikukawa, M., Pan, X., Fukui, H., Qi, Z. Int. J. Cancer, 78, 242-247, 1998), the L-plastin promoter (Chung, I., Schwartz, PE., Crystal, RC., Pizzorno, G, Leavitt, J., Deisseroth, AB. Cancer Gene Therapy, 6, 99-106, 1999), the arginine vasopressin promoter (Coulson, JM, Staley, J., Woll, PJ. British J. Cancer, 80, 1935-1944, 1999), the E2f promoter (Tsukada et al. Cancer Res., 62, 3428 - 3477), the uroplakine II promoter (Zhang et al., Cancer Res., 62, 3743-3750, 2002) and the PSA promoter (Hallenbeck PL, Chang, YN, Hay, C, Golightly, D., Stewart, D., Lin, J., Phipps, S., Chiang, YL. Human Gene Therapy, 10, 1721-1733, 1999). Furthermore, the YB-1 dependent E2-late promoter of adenoviruses as described in German patent application DE 101 50 984.7, is a promoter which can be used in the present invention.

It is known that the telomerase promoter is of crucial importance in human cells. Accordingly, telomerase activity is regulated through transcriptional control of the telomerase reverse transcriptase gene (hTERT), which is the catalytic subunit of the enzyme. The expression of the telomerase is active in 85 % of human tumor cells. In contrast thereto, it is not active in most of the normal cells. Exempt therefrom are germ cells and embryonic tissue (Braunstein, I. et al., Cancer Research, 61, 5529-5536, 2001; Majumdar, A. S. et al., Gene Therapy 8, 568-

578, 2001). More detailed studies on the hTERT promoter have revealed that fragments of the promoters 283 bp and 82 bp, respectively, distant from the initiation codon are sufficient for specific expression in tumor cells (Braunstein I. et al.; Majumdar AS et al., supra). Therefore, this promoter and the specific fragments, respectively, are suitable to provide for a specific expression of a gene and particularly of a transgene, preferably one of the transgenes disclosed herein, in tumor cells only. The promoter shall allow the expression of the modified oncogene, preferably the E1A oncogene protein, in tumor cells only. Also, in a preferred embodiment, the expression of a transgene, particularly one which is selected from the group comprising E4orf6, E1B55kD, ADP and YB-1, in such adenoviral vector is under the control of any of these promoters. It is also within the present invention that the open reading frame of the transactivating oncogene protein, in particular of the E1A protein, is in frame with one or several of the gene products of the adenoviral system. The open reading frame of the transactivating E1A protein, however, can also be independent therefrom.

The various transgenes, thus also E1B55kD, E4orf6, ADP and the like, in particular if they are viral genes, can, in principle, be cloned from any respective virus, preferably adenovirus and more preferably adenovirus Ad5. In the prior art furthermore a multitude of plasmids is described which contain respective genes and from which these may subsequently be taken and introduced into the adenoviruses of the present invention as well as into the viruses used in accordance with the present invention. An example for such a plasmid which expresses E1B55kD, is, for example, described by Dobbelstein, M. et al., EMBO Journal, 16, 4276-4284, 1997. The coding region of the E1B55K gene can be excised together with the 3' non-translating region (the 3'UTR region is preferably located at the base position 3507-4107 within the adenoviral wildtype genome), for example from this gene by Bam HI from plasmid pDCRE1B. The corresponding fragment comprising the E1B55kD gene as well as the 3' non-coding region corresponds to nucleotides 2019-4107 of adenovirus type 5. It is, however, also within the present invention that the E1B55kD gene is excised by means of restriction enzyme Bam HI and BfrI and XbaI, respectively, from said plasmid and is subsequently cloned into the adenovirus. It is also within the present invention that also analogs thereof and in particular analogs of the 3'UTR region are used within the present invention. An analog to the 3'UTR region is any sequence which has the same effect as the 3'UTR region, in particular the same effect in relation to the expression of a gene, preferably the E1B55kD gene. Such analogs may be determined by the one skilled in the art by means of routine experiments, for example by extending and truncating the 3'UTR region by one or several nucleotides and

subsequently testing whether the thus obtained analog still has the same effect as the 3'UTR region as described above. In an embodiment the term 3'UTR region thus also comprises any analog.

It is within the present invention that, if not indicated to the contrary, in case it is referred to viruses of the present invention it is referred to both the nucleic acid coding therefore as well as the adenoviral particles, preferably including the respective nucleic acid. The respective nucleic acid may also be present as being integrated in a different vector.

It is intended that with regard to the characteristics of the cells for the lysis of which the adenoviruses described herein are used in accordance with the present invention, these are, in an embodiment, resistant, preferably have a multidrug or multiple resistance. Resistance as used herein, refers preferably to a resistance against the cytostatics described herein. This multidrug resistance preferably goes along with the expression, preferably an overexpression, of the membrane-bound transport protein P-glycoprotein which can be used as a marker for determining respective cells and can thus also be used for tumors and respective groups of patients having such multidrug resistance. The term resistance as used herein comprises both the P-glycoprotein mediated resistance which is also referred to as classical resistance, as well as atypical resistance which comprises resistance which is mediated through MRP, or other, non-P-glycoprotein mediated resistances. A further marker, which correlates with the expression of YB-1, is topoisomerase II alpha. Insofar, in a screening for determining whether a patient may be treated with an expectation of success using the adenoviruses in accordance with the present invention, expression of topoisomerase II alpha can be used instead of or in addition to the determination of YB-1 in the nucleus. A further marker which can basically be used in a manner similar as P-glycoprotein, is MRP. A further marker, at least to the extent that the colorectal carcinoma cells or patients with colorectal carcinoma are concerned, is PCNA (engl. proliferating cell nuclear antigen) (Hasan S. et al., Nature, 15, 387-391, 2001), as, for example, described by Shibao K. et al. (Shibao K et al., Int. Cancer, 83, 732-737, 1999). Finally, the expression of MDR (multiple drug resistance) is a marker in the aforedescribed sense (Oda Y et al., Clin. Cancer Res., 4, 2273-2277, 1998), at least for breast cancer cells and osteosarcoma cells. A further possible marker, which can be used in accordance with the present invention, is p73 (Kamiya, M., Nakazatp, Y., J Neurooncology 59, 143-149 (2002); Stiewe et al., J. Biol. Chem., 278, 14230-14236, 2003).

It is thus a particular advantage of the present invention that also patients can be treated using the adenoviruses in accordance with the present invention, as described herein, which are otherwise deemed as being no longer treatable in the clinical sense and where a further treatment of the tumor disease according to the methods of the prior art is no longer possible with a reasonable expectation of success, in particular where the use of cytostatics is no longer reasonably possible and can no longer be successfully performed in the sense of influencing or reducing the tumor. The term tumor refers herein in general to each and any tumor or cancer disease which either contains YB-1 in the nucleus inherently or contains YB-1 in the nucleus, preferably independent from the cell cycle, as a consequence of realising exogenous measures as described herein.

Additionally, the viruses described herein can, in principle, be used for the treatment of tumors.

The tumours which can in particular be treated by the viruses described herein are preferably those tumours which are selected from the group comprising tumours of the nervous system, ocular tumours, tumours of the skin, tumours of the soft tissue, gastrointestinal tumours, tumours of the respiratory system, tumour of the skeleton, tumours of the endocrine system, tumours of the female genital system, tumours of a mammary gland, tumours of the male genital system, tumours of the urinary outflow system, tumours of the haematopoietic system including mixed and embryonic tumours. It is within the present invention that these tumours are in particular resistant tumours as in particular defined herein.

The group of tumors of the nervous system preferably comprises:

1. Tumors of the skull as well as of the brain (intracranial), preferably astrocytoma, oligodendroglioma, meningioma, neuroblastoma, ganglioneuroma, ependymoma, schwannoglioma, neurofibroma, haemangioblastoma, lipoma, craniopharyngioma, teratoma and chordoma;

2. Tumors of the spinal cord and of the vertebral canal, preferably glioblastoma, meningioma, neuroblastoma, neurofibroma, osteosarcoma, chondrosarcoma, haemangiosarcoma, fibrosarcoma and multiple myeloma; and

3. Tumors of the peripheral nerves, preferably schwannoglioma, neurofibroma, neurofibrosarcoma and perineural fibroblastoma.

The group of the ocular tumors preferably comprises:

1. Tumors of the eyelids and of the lid glands, preferably adenoma, adenocarcinoma, papilloma, histiocytoma, mast cell tumor, basal-cell tumor, melanoma, squamous-cell carcinoma, fibroma and fibrosarcoma;

2. Tumors of the conjunctiva and of the nictitating membrane, preferably squamous-cell carcinoma, haemangioma, haemangiosarcoma, adenoma, adenocarcinoma, fibrosarcoma, melanoma and papilloma; and

3. Tumors of the orbita, the optic nerve and of the eyeball, preferably retinoblastoma, osteosarcoma, mast cell tumor, meningioma, reticular cell tumor, glioma, schwannoglioma, chondroma, adenocarcinoma, squamous-cell carcinoma, plasma cell tumor, lymphoma, rhabdomyosarcoma and melanoma.

The group of skin tumors preferably comprises:

Tumors of the histiocytoma, lipoma, fibrosarcoma, fibroma, mast cell tumor, malignant melanoma, papilloma, basal-cell tumor, keratoacanthoma, haemangiopericytoma, tumors of the hair follicles, tumors of the sweat glands, tumors of the sebaceous glands, haemangioma, haemangiosarcoma, lipoma, liposarcoma, malignant fibrous histiocytoma, plasmacytoma and lymphangioma.

The group of tumors of the soft-tissues preferably comprises:

Tumors of the alveolar soft-tissue sarcoma, epithelioid cell sarcoma, chondrosarcoma of the soft-tissue, osteosarcoma of the soft-tissues, Ewing's sarcoma of the soft-tissues, primitive neuroectodermal tumors (PNET), fibrosarcoma, fibroma, leiomyosarcoma, leiomyoma, liposarcoma, malignant fibrous histiocytoma, malignant haemangiopericytoma, haemangioma, haemangiosarcoma, malignant mesenchymoma, malignant peripheral nerve sheath tumor (MPNST, malignant schwannoglioma,

malignant melanocytic schwannoglioma, rhabdomyosarcoma, synovial sarcoma, lymphangioma and lymphangiosarcoma.

The group of gastrointestinal tumors preferably comprises:

1. Tumors of the oral cavity and of the tongue, preferably squamous-cell carcinoma, fibrosarcoma, Merkel cell tumor, inductive fibroameloblastoma, fibroma, fibrosarcoma, viral papillomatosis, idiopathic papillomatosis, nasopharyngeal polyps, leiomyosarcoma, myoblastoma and mast cell tumor;

2. Tumors of the salivary glands, preferably adenocarcinoma;

3. Tumors of the oesophagus, preferably squamous-cell carcinoma, leiomyosarcoma, fibrosarcoma, osteosarcoma, Barrett carcinoma and paraoesophageal tumors;

4. Tumors of the exocrine pancreas, preferably adenocarcinoma; and

5. Tumors of the stomach, preferably adenocarcinoma, leiomyoma, leiomyosarcoma and fibrosarcoma.

The group of the tumors of the respiratory system preferably comprises:

1. Tumors of the nose and nasal cavity, of the larynx and of the trachea, preferably squamous-cell carcinoma, fibrosarcoma, fibroma, lymphosarcoma, lymphoma, haemangioma, haemangiosarcoma, melanoma, mast cell tumor, osteosarcoma, chondrosarcoma, oncocytoma (rhabdomyoma), adenocarcinoma and myoblastoma; and

2. Tumors of the lung, preferably squamous-cell carcinoma, fibrosarcoma, fibroma, lymphosarcoma, lymphoma, haemangioma, haemangiosarcoma, melanoma, mast cell tumor, osteosarcoma, chondrosarcoma, oncocytoma (rhabdomyoma), adenocarcinoma, myoblastoma, small-cell carcinoma, non-small cell carcinoma, bronchial adenocarcinoma, bronchoalveolar adenocarcinoma and alveolar adenocarcinoma.

The group of the skeleton tumors preferably comprises:

osteosarcoma, chondrosarcoma, parosteal osteosarcoma, haemangiosarcoma, synovial cell sarcoma, haemangiosarcoma, fibrosarcoma, malignant mesenchymoma, giant-cell tumor, osteoma and multilobular osteoma.

The group of the tumors of the endocrine system preferably comprises:

1. Tumors of the thyroid gland/parathyroid, preferably adenoma and adenocarcinoma;

2. Tumors of the suprarenal gland, preferably adenoma, adenocarcinoma and pheochromocytoma (medullosuprarenoma);

3. Tumors of the hypothalamus/hypophysis, preferably adenoma and adenocarcinoma;

4. Tumors of the endocrine pancreas, preferably insulinoma (beta cell tumor, APUDom) and Zollinger-Ellison syndrome (gastrin secernent tumor of the delta cells of the pancreas); and

5. as well as multiple endocrine neoplasias (MEN) and chemodectoma.

The group of the tumors of the female sexual system tumors preferably comprises :

1. Tumors of the ovaries, preferably adenoma, adenocarcinoma, cystadenoma, and undifferentiated carcinoma;

2. Tumors of the uterine, preferably leiomyoma, leiomyosarcoma, adenoma, adenocarcinoma, fibroma, fibrosarcoma and lipoma;

3. Tumors of the cervix, preferably adenocarcinoma, adenoma, leiomyosarcoma and leiomyoma;

4. Tumors of the vagina and vulva, preferably leiomyoma, leiomyosarcoma, fibroleiomyoma, fibroma, fibrosarcoma, polyps and squamous-cell carcinoma.

The group of tumors of the mammary glands preferably comprises:

fibroadenoma, adenoma, adenocarcinoma, mesenchymal tumora, carcinoma, carcinosarcoma.

The group of the tumors of the male sexual system preferably comprises:

1. Tumors of the testicles, preferably seminoma, interstitial-cell tumor and Sertoli cell tumor;

2. Tumors of the prostate, preferably adenocarcinoma, undifferentiated carcinoma, squamous-cell carcinoma, leiomyosarcoma and transitional cell carcinoma; and

3. Tumors of the penis and the external gentials, preferably mast cell tumor and squamous-cell carcinoma.

The group of tumors of the urinary outflow system preferably comprises:

1. Tumors of the kidney, preferably adenocarcinoma, transitional cell carcinoma (epithelial tumors), fibrosarcoma, chondrosarcoma (mesenchymal tumors), Wilm`s tumor, nephroblastoma and embryonal nephroma (embryonal pluripotent blastoma);

2. Tumors of the ureter, preferably leiomyoma, leiomyosarcoma, fibropapilloma, transitional cell carcinoma;

3. Tumors of the urinary bladder, preferably transitional cell carcinoma, squamous-cell carcinoma, adenocarcinoma, botryoid (embryonal rhabdomyosarcoma), fibroma, fibrosarcoma, leiomyoma, leiomyosarcoma, papilloma and haemangiosarcoma; and

4. Tumors of the urethra, preferably transitional cell carcinoma, squamous-cell carcinoma and leiomyosarcoma.

The group of tumors of the haematopoietic system preferably comprises:

1. Lymphoma, lymphatic leukemia, non-lymphactic leukemia, myeloproliferative leukemia, Hodgkin´s lymphoma, Non-Hodgkin`s lymphoma.

The group of the mixed and embryonal tumors preferably comprises:

Haemangiosarcoma, thymoma and mesothelioma.

Preferably, these tumors are selected from the group comprising breast cancer, ovary carcinoma, prostate carcinoma, osteosarcoma, glioblastoma, melanoma, small-cell lung carcinoma and colorectal carcinoma. Further tumors are those which are resistant as described herein, preferably those which are multiple resistant, particularly also those tumors of the group described above.

The invention is related in a further aspect to a method for the screening of patients which can be treated using one of the modified adenoviruses, i. e. an adenovirus as used in accordance with the present invention such as, for example, AdΔ24, dl922-947, E1Ad/01/07, CB016 or the viruses described in European patent EP 0 931 830, whereby such method comprises the following steps:

- examining a sample of a tumor tissue and
- determining whether YB-1 is located in the nucleus independent from the cell cycle.

The presence of the afore-described markers can be detected instead of or in addition to YB-1.

In case that the tumor tissue or a part thereof comprise YB-1 in the nucleus, in particular independent from cell cycle, the adenoviruses disclosed therein, can be used in accordance with the practice of the present invention.

In an embodiment of the method according to the present invention the examination of the tumor tissue is done by using an agent which is selected from the group comprising antibodies against YB-1, aptamers against YB-1 and spiegelmers against YB-1 as well as anticalines against YB-1. Basically, the same means can be produced for the corresponding markers and

used accordingly. The manufacture of antibodies, in particular monoclonal antibodies, is known to the ones skilled in the art. A further means for specific detection of YB-1 or the markers, are peptides which bind with a high affinity to the target structures, in the present case YB-1 or said markers. In the prior art methods are known such as phage-display in order to generate such peptides. Typically, a peptide library is taken as a starting point, whereby individual peptides have a length of from 8 to 20 amino acids and the size of the library is about 10^2 to 10^{18}, preferably 10^8 to 10^{15} different peptides. A special form of target molecule binding polypeptides are the so-called anticalines which are, for example, described in German patent application DE 197 42 706.

A further means for specific binding of YB-1 or the corresponding markers disclosed herein and thus for the detection of cell cyclus independent localisation of YB-1 in the cellular nucleus, are the so-called aptamers, i.e. D-nucleic acids which are present either as RNA or DNA either as a single strand or a double strand and specifically bind to the target molecule. The generation of aptamers is, for example, described in European patent EP 0 533 838. A special form of aptamers are the so-called aptazymes, which, for example, are described by Piganeau, N. et al. (2000), Angew. Chem. Int. Ed., 39, no. 29, pages 4369 – 4373. These are special embodiments of aptamers insofar as they comprise apart from the aptamer part a ribozyme part and get catalytically active upon binding or release of the target molecule binding to the aptamer part and cleave a nucleic acid substrate which goes along with the generation of a signal.

A further form of aptamers are the so-called spiegelmers, i. e. target molecule binding nucleic acids which are made of L-nucleic acids. The method for the manufacture of such spiegelmers is, for example, described in WO 98/08856.

The sample of the tumor tissue can be obtained by puncture or through surgery. The assessment whether YB-1 is localised in the nucleus independent from the cell cycle, is frequently done by using microscopic techniques and/or immuno histoanalysis, preferably using the antibody or any of the other aforementioned means. Further means for detecting YB-1 in the nucleus and in particular for detecting that YB-1 is located there independent from the cell cycle, are known to the one skilled in the art. For example, the localisation of YB-1 can be easily detected in stained tissue sections when screening them. The frequency of the presence of YB-1 in the nucleus already indicates that the localisation is independent from

the cell cycle. A further option for cell cycle independent detection of YB-1 in the nucleus resides in the staining against YB-1 and detection whether YB-1 is localised in the nucleus and determination of the phase of the cells. This as well as the detection of YB-1 may also be performed by using the aforementioned means directed against YB-1. The detection of the means is done by methods known to the one skilled in the art. By said agents specifically binding to YB-1 and not to any other structures within the sample to be analysed, particularly the cells, their localisation and because of their specific binding to YB-1 also the localisation of YB-1 can be detected and established by a suitable labelling of the means. Methods for the labelling of said means are known to the ones skilled in the art.

It is within the present invention that the viruses described herein, whether they are the viruses of the present invention or whether they are the viruses to be used in accordance with the present invention, are also used in connection with diseases, preferably tumor diseases and more preferably tumor diseases where at least a part of the tumor cells exhibits a multiple resistance, in particular a multi-drug resistance, in which YB1 is deregulated. This applies also to each and any other aspect as described herein in connection with the cells and tumors to the extent that it refers to cells and diseases where YB1 is present in the nucleus, preferably independent of the cell cycle.

In the following, the present invention shall be further illustrated by reference to the figures and samples from which new features, embodiments and advantages may be taken.

Fig. 1 shows the structural design of the adenoviral vectors referred to as AdE1/E3-minus herein which are E1/E3-deleted adenoviruses, of wildtype adenovirus and adenovirus dl520.

Fig. 2 shows the binding domains of the E1A protein with regard to the binding of p300, p107 and p105.

Fig. 3 shows U2OS cells which do not have YB-1 in the nucleus, after infection with the E1/E3-deleted adenoviruses Ad5, referred to as E1/E3-minus Ad5, and dl520.

Fig. 4 shows 257RDB cells which have YB-1 in the nucleus, after infection with the E1/E3-deleted adenoviruses Ad5, referred to as E1/E3-minus Ad5, and adenovirus dl520.

Fig. 5 shows 257RDB cells and U2OS cells after infection with adenovirus dl1119/1131.

Fig. 6 shows the result of an EMSA analysis which confirms that YB-1 is present in multidrug resistant cells and cell lines 257RDB, 181 RDB, MCF-7Ad, respectively, whereas YB-1 is not present in the nucleus of U2OS and HeLa cells.

Fig. 7 shows the structural design of the E1A protein of wildtype adenovirus, of adenovirus dl520 and adenovirus dl1119/1131.

Fig. 8 is a column diagram showing the replication efficiency of adenoviruses in the presence of additionally expressed viral proteins in absolute figures.

Fig. 9 is a column diagram showing the increase of replication efficiency of adenoviruses in the presence of additionally expressed viral proteins.

Fig. 10 shows wells grown with U2OS cells after crystal violet staining and infection with dl520 with 10 and 30 pfu/cell, respectively, and control (K) without administration of daunorubicine and with the administration of 40 ng daunorubicine per ml, respectively.

Fig. 11 shows wells grown with HeLa cells, after crystal violet staining and infection with dl520 and 10 and 30 pfu/cell and control (K), respectively, without administration of daunorubicine and administration of 40 ng daunorubicine per ml, respectively.

Fig. 12 is a diagram of the tumor volume of tumors having different origins (RDB257 and HeLa) as a function of time after treatment with PBS and dl520, respectively.

Fig. 13 show pictures of sacrificed mice which developed a tumor based on RDB257 cells after treatment with PBS and 5 x 10^8 pfu dl520, respectively.

Fig. 14 is the result of a Southern Blot analysis of a cell extract (of the tumors grown subcutaneously) of RDB257 cells and HeLa cells after infection with dl520.

Fig. 15 is a column diagram showing the replication efficiency and particle formation, respectively, of dl520 and wildtype adenoviruses in YB-1 nucleus-positive tumor cells (257RDB and 181RDB) and YB-1 nucleus-negative tumor cells (HeLa, U2OS).

Fig. 16 shows the structural design of wildtype adenovirus and adenoviral vector AdXvir03.

Fig. 17 shows the structural design of adenoviral vector AdXvir03/01.

Fig. 18A/B shows wells grown with 181RDB cells (Figs. 18A) and 272RDB cells (Fig. 18B) after crystal violet staining and infection with Ad312 (20 pfu/cell), Xvir03 (5 pfu/cell) and control (non-infected), whereby crystal violet staining was performed five days past infection.

Fig. 19 shows the result of a Southern blot analysis of the replication behaviour of adenovirus dl 520 in U373 cells with and without treatment of the cells with irinotecan.

Fig. 20 shows the result of a Southern blot analysis of the replication behaviour of adenovirus dl 520 in U373 cells with and without treatment of the cells with trichostatin A.

Fig. 21 shows the result of a FACS analysis of U 373 cells treated with trichostatin with regard to the expression of coxsackievirus-adenovirus receptor (CAR), represented as percentage of CAR-positive cells.

Fig. 22 shows four different panels of cell layers for illustrating the effect of replicating adenovirus dl 520 and irinotecan and trichostatin in various combinations;

Fig. 23 shows a schematic representation of the ORF of E1B55K with the 3'UTR fragment and the restriction cleavage site Bfr I at position 3532.

Fig. 24 shows the sequence of the E1B55k 3'UTR region corresponding to sequence position 3507 to 4174 of wildtype Ad 5.

Example 1: Types of E1A modifications as may be comprised by the adenoviruses which are used in accordance with the invention

Fig. 1 shows the structural design of adenoviral vectors AdE1/E3-minus, i. e. E1/E3-deleted adenoviruses, wildtype adenovirus and adenovirus dl520.

Adenovirus AdE1/E3-minus does not have a region coding for a functional E1A or a functional E1B or E3 and is used in the present experiments as a control for toxicity.

Wildtype E1A gene codes for a total of 5 proteins which are generated through alternative splicing of the E1A RNA. Among others, two different proteins are generated, namely a 289 amino acid protein and a 243 amino acid protein. dl520 does not code for the 289 amino acid protein as it has a deletion in the CR3 stretch of the E1A gene which results in the lack of the 13S gene product. The adenovirus dl520 which may be used in accordance with the invention is referred to as 12S-E1A virus by those skilled in the art. Adenovirus dl347 (Wong und Ziff, J. Virol., 68, 4910-4920, 1994) known in the prior art is also a 12S-E1A virus which can be used in accordance with the present invention.

Within the 289 amino acid protein which is encoded by the 13S-E1A mRNA, there are 3 regions which are conserved among various adenoviral subtypes. These are referred to as CR1, CR2 and CR3. While CR1 and CR2 are present in both E1A proteins (E1A 12S and E1A 13S), i. e. in both the 289 amino acid and the 243 amino acid protein, the CR3 region is only present in the bigger one of the two aforementioned proteins.

The CR3 region is required for the activation of viral genes, in particular of E1B, E2, E3 and E4. Viruses which only comprise the smaller, i. e. 243 amino acid protein are only very weakly transactivating the viral genes and do not promote adenoviral replication in those cells which do not have YB-1 in the nucleus. As YB-1 is present in the nucleus only in tumor cells and can be detected only there, this vector is suitable to induce tumor-specific replication.

Due to the deletion of CR3 in dl520 this adenovirus cannot translocate cellular YB-1 into the cell's nucleus which is also referred to herein as translocation, and is thus not in a position to replicate in cells which are YB-1 nucleus-negative and is thus a virus which can be used in accordance with the present invention, whereby this virus comprises the transactivation required in accordance with the present invention.

Example 2: Mode of action of adenoviruses in depending on the Rb status of cells

Fig. 2 shows the binding domains of the E1A protein with regard to the binding of p300, p107 and p105. P300, as well as p107, is a cellular binding protein. The binding of the retinoblastoma protein (pRb), a tumor suppressor protein, is mediated through CR1 and CR2. Studies have shown that pRb and p107/p300 are in combination with the cellular transcription factor E2F effective in regulating transcription. The wildtype E1A protein interferes with the binding of E2F to Rb. The thus released E2F binds to the E2 early promoter and induces adenoviral replication thereby.

It is known from the prior art that certain deletions in the E1A oncoprotein may result in recombinant adenoviral vectors such as those mentioned in the following, which are capable of replicating predominantly in Rb-negative cells and can be used in accordance with the present invention. For example, the adenoviral vector dl922-947 comprises a deletion in the CR2 region (amino acid positions 122-129) and the vector CB016 has deletions in the CR1 region (amino acid positions 27-80) and CR2 region (amino acid positions 122-129). The vector E1A*dl*01/07 comprises a deletion in the CR2 region (amino acid positions 111-123). Additionally, because of an additional deletion at the N-terminus (amino acid positions 4-25), additionally, there is no binding to protein p300. The adenoviral vector AdΔ24 comprises a

deletion in the CR2 region (amino acid positions 120-127). The adenoviral vector described in patent EP 0 931 830 comprises deletions in the CR1 region and CR2 region.

The binding mechanism of E2F/RB and the release of E2F mediated through E1A is fundamentally different from the mechanism underlying the present invention. Unlike assumed in the prior art it is not the release of E2F from the Rb protein which is essential, not to say critical for viral replication, but it is the nuclear localisation of the human transcription factor YB-1. This transcription factor is, in normal cells, only present in the cytoplasm over most of the cell cycle. After infection with an adenovirus it is induced into the nucleus under certain circumstances or is already present in the nucleus in distinct cellular systems, such as distinct tumor diseases including, for example, but not limited thereto, breast cancer, ovary carcinoma, prostate carcinoma, osteosarcoma, glioblastoma, melanoma, small cell lung carcinoma and colorectal carcinoma.

Example 3: Infection of U2OS cells

100,000 U2OS cells were plated per well. On the next day the cells were infected with the various adenoviruses as depicted in Fig. 3. The infection was performed in 500 µl serum free DMEM medium at 37° C for 1 h. Subsequently, the infection medium was removed and replaced by 2 ml complete medium (10 % FCS/DMEM). The analysis was performed after 3 days using crystal violet staining.

As may be taken from Fig. 3, the U2OS cells which do not have YB-1 in the nucleus, show no lysis as illustrated by crystal violet staining after infection with two different adenoviruses, namely the E1/E3-deleted adenovirus referred to as E1/E3-minus, and adenovirus dl520, which can be used in accordance with the present invention. In connection therewith, first, the medium is removed. Subsequently, the cells are overlaid with crystal violet (50 % ETOH, 3 % formaldehyde, 5 % acetic acid, 1 % crystal violet) and incubated at room temperature for 5-10 min. Subsequently, the plates having 6 wells are thoroughly rinsed with water and dried at room temperature.

This confirms the finding underlying the present invention that the presence of YB-1 is required in order to induce the viruses used in accordance with the present invention, to lyse the infected cells.

Example 4: Infection of 257RDB cells

100,000 257RDB cells were plated per well. On the next day the cells were infected with the various adenoviruses as depicted in Fig. 4. The infection was performed in 500 µl serum free DMEM medium for 1 h at 37° C. Subsequently, the infection medium was removed and replaced by 2 ml complete medium (10 % FCS/DMEM). The analysis was performed after three days using crystal violet staining.

The result of this experiment is depicted in Fig. 4. The adenovirus referred to as E1/E3-minus Ad5 which is E1/E3-deleted, did not show any lysis at low MOIs (pfu/cell) upon infection of 257RDB cells which have YB-1 in the nucleus. In contrast thereto, dl520 which, as shown in example 3, does not replicate in YB-1 nucleus-negative cells and at the same time codes with E1A for a transactivating oncogene protein in accordance with the present invention, results in a factually complete lysis at an MOI (multiplicity of infection) of 40 pfu per cell and a still predominant lysis at an MOI of 10 pfu per cell. It can be concluded therefrom that dl520 and similar viruses such as described herein by dl1119/1131 or AdXvir 03, require an MOI which is reduced by about 1 magnitude (factor of ten) compared to E1-deleted or an E1/E3-deleted adenovirus which justifies their clinical use.

As depicted in Fig. 7, the protein E1A of dl520 is characterised in that the CR3 region thereof is deleted which results in the transactivation required for the use in accordance with the present invention and replication in YB-1 nucleus-positive cells.

Example 5: Infection of 257RDB and U2OS cells with dl1119/1131

As depicted in Fig. 5, there is no lysis at an MOI of 20 pfu per cell upon infection of YB-1 nucleus-negative U2OS cells with adenovirus dl1119/1131 which exhibits a deletion of amino acids 4-138 of the E1A protein and the nucleic acid coding therefor, and further comprises a

stop codon after amino acid 218, whereby the expressed truncated E1A protein comprises the CR3 region of the complete E1A protein. As a negative control a non-infected cell layer was used.

In contrast thereto, there was factually a complete lysis of the cell layer at an MOI of 20 pfu per cell under the influence of adenovirus dl1119/1131 in a cellular system such as 257RDB which contains YB-1 in the nucleus, i. e. is YB-1 nucleus-positive. Insofar this example is another proof that a modified E1A oncogene protein which, as depicted in Fig. 7, comprises, for example, only the CR3 region and which is lacking the CR1 region and CR2 region, provides for the required transactivation in YB-1 nucleus-positive cells which is required for the replication of adenoviruses in accordance with the present invention, which results in viral replication. The adenovirus dl1119/1131 is thus a further adenovirus which can be used in accordance with the present invention. It is within the present invention that also viruses can be used which are designed similar to dl1119/1131 with regard to the CR3 region, but, in contrast thereto, have the CR1 region and/or CR2 region.

Example 6: Detection of nuclear YB-1 in multidrug resistant cells

The example is based on the consideration that nuclear YB-1 should bind as a transcription factor to the Y-box (CAAT sequence) within the mdr1 promoter (engl. multiple drug resistance promoter). In order to detect this, a so-called EMSA analysis (electrophoretic mobility shift assay) was performed. In connection therewith, nuclear protein is isolated and subsequently 1-10 µg protein is incubated together with a short DNA fragment (oligo) at 37° C. In order to determine nuclear YB-1, the following oligonucleotide was used: *mdr1* promoter in contrast to U2O3 (Position –86 to –67): TGAGGCTGATTGGCTGGGCA (the X-box is underlined).

This DNA fragment is radioactively labelled at the 5' end with ^{32}P prior to that. Subsequently, separation is performed in a native polyacryl amide gel. In case the protein YB-1 is binding to a sequence in the oligonucleotide, this can be detected as any non-bound oligonucleotide is migrating faster in the gel than bound oligonucleotide (Holm, P. S. et al., JBC 277, 10427-10434, 2002; Bargou, R. C. et al., Nature Medicine 3, 447-450, 1997).

As depicted in Fig. 6, it could be shown with the EMSA analysis that YB-1 is present in the nucleus of multidrug resistant cells 257RDB, 181RDB and MCF-7Ad cells in contrast to cell lines U2OS and HeLa cells.

The results shown in example 4 and 5 confirm that the adenoviruses dl520 and dl1119/1131 replicate in YB-1 nucleus-positive cells such as, e. g., 257RDB in contrast to U205, and induce lysis thereof. This confirms the finding about the use of the adenoviruses in accordance with the present invention. Additionally, the results confirm that already a, compared to wildtype adenovirus, weak transactivation of viral genes in YB-1 nucleus-positive cells through modified or deleted E1A gene products results in successful replication and lysis of such cells in the presence of YB-1 in the nucleus, including, for example, multidrug resistant cells and that the adenoviruses as described herein, can thus be used in the lysis of such tumors.

Example 7: Increase of replication efficiency of E1-minus adenoviruses

This example shows that the early viral genes E1B-55K and E4orf6 can be substituted through transfection with the plasmid pE4orf6 and infection with the E1/E3-deleted adenovirus Ad-55K. Ad-55K is an E1/E3 deleted virus, whereby E1B-55K is cloned into E1 and is under the control of CMV (Dobbelstein, M. et al., EMBO Journal, 16, 4276-4284, 1997). This substitution is necessary with regard to the fact that AdYB-1, i. e. an adenovirus which expresses YB-1, does not express these early genes and that the present inventor has recognised that a substitution of these early genes in a replication system which contains YB-1 in the nucleus, is capable of increasing replication efficiency and particle formation efficiency, respectively, to an extent comparable to the one of wildtype adenoviruses of type Ad5.

The following was done:

Transfection of each 10^5 U2OS cells with the plasmid pE4orf6 using lipofectamine. The plasmid pE4orf6 carries the DNA sequence coding for the early viral gene E4orf6 under the control of CMV.

24 h after transfection with the plasmid pE4orf6 the cells were infected with the YB-1 expressing E1/E3-deleted adenovirus AdYB-1 (50 pfu/cell) and the E1/E3-deleted E1B-55K adenovirus Ad-55K (50 pfu/cell). Ad-55K is an E1/E3-deleted virus which carries as transgene the viral gene E1B-55K under CMV control.

Subsequently, the cells were removed from the medium (2 ml) 5 days after infection (= post infectionem). The release of the viral particles from the isolated cells was done by alternating freezing and thawing for three times (thaw/freeze). Subsequently, a plaque assay was performed on 293 cells for determining the generated infectious particles (plaque forming units per ml (pfu/ml)). The result is depicted in Figs. 8 and 9. Figs. 8 shows the result of the plaque assay, represented in absolute figures. The most significant difference compared to infection with AdYB-1 alone is shown by transfection with the plasmid pE4orf6 and co-infection with the two viruses AdYB-1 and Ad-55K. Fig. 9 shows the result of Fig. 8, whereby the increase of the replication efficiency is represented as multifold of the replication determined for AdYB-1. The cells infected with plasmid pE4orf6 and subsequently with AdYB-1 and E1B-55K (Ad-55K) produced up to 25 times more pfu/ml.

Based on these results it can be concluded that the substitution of E1B-55K and E4orf6 increases the number of viruses formed (pfu/ml) after infection with the E1/E3-deleted adenovirus AdYB-1 by a factor of up to 25. The additive effects of E1B-55K and E4orf6 on the production of plaque forming units (pfu) is significantly higher compared to the effects of each of the two gene products.

Control experiments with one plasmid which expresses EGFP, clearly showed that in the experimental approach chosen only 10 % of the cells were successfully transfected with plasmid pE4orf6. The number of the particles formed in the cells which express both E1B-55K and E4orf6 is comparable to the one of human adenovirus type 5 (wildtype). This confirms the finding underlying the present invention that the expression of E4orf6 and E1B-55K is, in combination with the nuclear localisation of YB-1, able to provide for adenoviral replication and particle formation, in particular of E1A-deleted adenoviruses, which is comparable to the one of wildtype Ad5.

Example 8: Increased replication of adenoviruses which are not replicating in YB-1 nucleus-negative cells, in YB-1 nucleus-positive cells upon administration of cytostatics

It is known in the prior art that the addition of different cytostatics induces nuclear localisation of the human transcription factor YB-1. As has been found by the present inventor, YB-1 localised in the nucleus controls adenoviral replication by means of activation of the adenoviral E2-late promoter. The combination of both effects can be used in order to provide for specific tumor lysis.

In the practising of the oncolytic assays the following procedure was followed: 200,000 cells (HeLa and U2OS, respectively) were plated into each well of a 6 well plate. On the next day 40 ng/ml (final concentration) of daunorubicine were added. After 3 hours of incubation the cells were infected with 10 and 30 pfu dl520/cell, respectively. Subsequently, the cells were incubated in cytostatic free medium. After 3 - 5 days the cells were stained using crystal violet.

As may be taken from Fig. 10 and 11, the addition of daunorubicine induces the replication of dl520 through nuclear localisation of YB-1. Thus, dl520 creates a bigger tumorlytic effect in combination with the cytostatic daunorubicine compared to daunorubicine alone.

Example 9: In vivo tumor lysis by dl520

The HeLa (YB-1 nucleus-negative) and 257RDB (YB-1 nucleus-positive) cells used in this in vivo study, were expanded under sterile cell culture conditions. Prior to the injection of the cells into mice (strain CD1NuNu) in order to generate a subcutaneous tumor, the cells are harvested by trypsinisation, taken up in DMEM medium (10 % FCS), counted and washed with PBS one time. Subsequently, the cells are centrifuged, the PBS aspired and the cells are portioned in fresh PBS with the desired cell number. The cell number which was subcutaneously injected in this study, was each 5 x 10^6 cells of both cell lines. The injection was performed subcutaneously into one flank of the animals, whereby HeLa cells were injected into the right side and 257RDB cells were injected into the left side for better distinction. The growth of the tumors was controlled twice a week and thereby the length and

the width of the tumors was measured using vernier calipers. Based thereon, the tumor volume was calculated based on the following mathematical formula:

$$3/4\pi * a/2 * (b/2)^2 \quad a = \text{length}, b = \text{width}$$

Once the tumor has reached a volume of 200 to 520 mm^3, the virus and PBS as negative control, respectively, were intratumorally applied. The volumes to be injected were identical and were 50 µl each time. This was repeated on 3 consecutive days. The overall dosage of applied viruses was 5×10^8 pfu. Subsequently, the tumor growth was continued to be documented twice a week and the volume was calculated. At the end of the study the mice were sacrificed and the tumors removed for further analysis.

The results are depicted in figures 12 and 13.

Fig. 12 shows a diagram representing the tumor volume as a function of time and the various treatment schemes. In case the tumor was formed by RDB257, there was a significant growth of the tumor to about 438 mm^3 to 1466 mm^3 upon injection of PBS. Under the influence of the vector dl520 which was used in accordance with the invention, tumor growth could be reduced significantly. Starting from a mean tumor size of 344 mm^3, the tumor size increased only by 21 % to a total of 543 mm^3.

In the present example the tumor consisting of HeLa cells was used as a control which upon administration of PBS behaved similarly to the RDB257 based tumor upon administration of PBS. Tumors based on HeLa cells and treated with dl520, however, still showed a significant increase in tumor growth starting from 311 mm^3 and increasing to 1954 mm^3.

Fig. 13 shows a picture of the sacrificed nude mice which had a tumor grown using RDB257. It can be clearly seen that after the application of adenovirus dl520 in accordance with the present invention a significant reduction of the tumor occurred. In the present case there was even a reduction in the tumor volume (day 1 after administration of virus dl520: 515 mm^3; day 30 after administration of virus dl520: 350 mm^3).

Example 10: Southern Blot of tumor DNA

DNA was extracted from a tumor sample which has been taken from the middle of the tumor developed in example 9. For isolation the Dneasy Tissue Kit of Qiagen is used. The DNA isolation is done in accordance with manufacturer's instructions. In accordance therewith, the DNA was released from the cells through alkaline lysis. Subsequently, the isolated DNA is purified over a column. Subsequently, the concentration of the isolated DNA is determined by photometry at 260 nm. The analysis was performed using 2 µg of the DNA samples which were digested with 10 units of restriction enzyme Kpn I. Subsequently, an electrophoretic separation of the samples was performed in a 0.8 % agarose gel. Subsequently, the DNA was blotted onto a nylon membrane (performed according to the system of Schleicher & Schuell). The DNA blotted onto the membrane is hybridised against a specific 1501 bp DNA probe. The 1501 bp DNA probe specifically binds to the 3369 bp Kpn I fragment within the E2A coding Ad5 sequence. The probe was prepared by PCR (primer: 5'- GTC GGA GAT CAG ATC CGC GT, 5'- GAT CCT CGT CGT CTT CGC TT) and radioactively labelled using ^{32}P. Subsequently, the membrane is washed and exposed to a film.

The result of the Southern Blot of tumor DNA is depicted in Fig. 14. The analysis confirms that only dl520 replicates in vitro in resistant cells RDB257, as depicted in lanes 3, 4 and 5. Lane 1 shows as positive control Ad-5d, lane 6, 7 and 8 show DNA from HeLa cells which were infected with dl520. As HeLa cells are not YB-1 nucleus positive the virus dl520 did not replicate so that, in accordance therewith, the E2A sequence could not be detected.

A further result with dl520 is depicted in Fig. 15. Based on a plaque assay the particle formation (pfu/ml) was investigated after infection with dl520 and wildtype adenovirus. Various YB-1 nucleus-positive (257RDB and 181RDB) tumor cells and YB-1 nucleus-negative tumor cells were infected with dl520 and wildtype adenovirus.

The following procedure was practiced:

100,000 – 200,000 cells each were plated in so-called plates having 6 wells (engl. 6 well plates) in L 15 medium (resistant cells) and DMEM (non-resistant cells) having 10 % FCS. After 24 h infection with dl520 and wildtype adenoviruses (10 pfu/cell) was performed. 3 days after infection (post infectionem) the viral particles were released from the cell

suspension (3 ml) by alternating freezing and thawing for three times. Subsequently, a plaque assay was performed on 293 cells for determining the formed infectious particles (plaque forming units per ml (pfu/ml)). The result is depicted in Fig. 15. The result of the plaque assay shows that dl520 is replicating in YB-1 nucleus-positive cells (257RDB and 181RDB) similar to wildtype adenovirus. Insofar a replication efficiency can be observed similar to the one of wildtype adenoviruses when using, in accordance with the present invention, the adenoviruses described herein.

Example 11: Structural design of the adenoviral vector Xvir03

Fig. 16 shows the structural design of the adenoviral vector Xvir03. The adenovirus Xvir03 is a so-called E1/E3-deleted adenovirus. This means that no E1A, E1B (E1B55k and E1B19K proteins) and E3 proteins are manufactured which are functional in adenoviral replication. The deletion of the E1 region extends from 342 – 3528; the deletion of the E3 region of the base position 27865 – 30995. As used herein, the term "E1-deleted virus" means a virus in which E1 is no longer functionally active. This can be achieved by inactivation with an otherwise mostly intact nucleic acid and amino acid sequence, respectively, however, can also mean a deletion of the E1 region coding proteins having various sizes. Because of the lack of the E1A and E1B protein and the nucleic acids coding therefor, the E4 region, such as E4orf6, is only weakly expressed (about 1 – 5 % compared to wildtype adenoviruses) or expressed not at all. The viral genes E1B55k and E4orf6 are expressed in the E1 region by means of the heterologuous CMV promoter (Clontech: Plasmid pShuttle) introduced into Xvir03. Instead of the CMV promoter each and any of the promoters as disclosed herein in connection with the expression of E1A can be used. The open reading frames of both genes are linked with each other by means of a so-called IRES sequence (engl. internal ribosomal entry site) (Pelletier, J. and Sonenberg, N. Nature, 1988, 334, 320 – 325). This element (Novagen: pCITE) provides for the expression of 2 proteins from one mRNA.

The vector was manufactured as follows: System Adeno-X of the company Clontech

The plasmid E1B55k-pShuttle was created by cloning the open reading frame of E1B55k from pCGNE1B from M. Dobelstein (University of Marburg) with XbaI and BfrI and BamHI only, respectively, into the pShuttle vector from Clontech, whereby in this case the ends are

made blunt ended and cloned into the blunt ended pShuttle. Subsequently, E1B55k in pShuttle was linearised with ApaI, the ends blunt ended and cut with NheI.

In a second vector, pcDNA3.1(+) (Invitrogen), subsequent to each other, the IRES element as a PCR product was cloned with pCITE-4a(+) of the company Novagen as template by means of TA cloning into the EcoRV cleaving site, and the E4orf6 from the plasmid pCMV-E4orf6 (M. Dobelstein, University of Marburg) was cloned by means of BamHI = IRES-E4orf6-pcDNA3.1(+). IRES-E4orf6 in pcDNA3.1(+) was linearised with NotI, the ends blunt ended and subsequently the fragment IRES-E4orf6 was cut out with NheI. The fragment IRES-E4orf6 was linked with the open vector E1B55k-pShuttle (blunt, NheI). The cassette was subsequently cloned from the E1B55k-IRES-E4orf6-pShuttle together with the CMV promoter and the *bovine growth hormone* (BGH)-PolyA into the ΔE1, ΔE3 Adeno-X-Plasmid (Clontech) with I-Ceu I and PI-SceI, and referred to as AdcmvE1B/IRES/E4orf6. Subsequently, the adenovirus was prepared in accordance with manufacturer's instructions (Clontech). The adeno plasmid which was linearised with PacI having the expression element CMV-E1B55k-IRES-E4orf6-BGH polyA was transfected into HEK293 cells and 11 days *post transfectionem* the ablating cells were removed together with the medium in order to release the generated adenoviruses through repeated freeze-thaw cycles.

It is within the present invention and feasible for the one skilled in the art with regard to the technical teaching provided herein, that other systems such as the system AdEasy of QBIOGENE and Microbix may be used for the manufacture of the adenoviruses according to the present invention, preferably the recombinant adenovirus, in particular those which contain, individually and/or together, the cassettes E4orf6-IRES-E1B55k and YB-1-IRES-E1A12S. Additionally, individual transgenes may be exchanged between the cassettes. It is within the present invention that also such adenoviruses can be manufactured and used in accordance with the present invention, where the cassette has the following design: E1B55k-IRES-E4orf6 and E1A12S-IRES-YB1.

In connection with the present invention a so called E1/E3 deleted recombinant adenovirus was used which contains the cassette E4orf6-IRES-E1B55k. It is, however, within an embodiment that the virus comprises only an E1-deletion, which means that the E3-region remains intact. Optionally, the E4-region may be partially and/or completely deleted.

In the manufacture of the vector using different systems it was proceeded as follows.

Manufacture of the adenovirus Ad-Xvir 3'UTR having an intact E3-region with the vector system according to Graham (company Microbix).

Cloning of the vector CMV-E4ORF6-IRES-E1B55k 3'UTR-polyA in pDelta E1sp1A

For the plasmid E1B55k 3'UTR-pShuttle (Clontech) the open reading frame having the 3'-UTR was prepared by amplification from the DNA of adenovirus type 5 (E1B55k forward primer = 5'-ATGGAGCGAAGAAACCC-3' and E1B55k 3'UTR backward primer = 5'-CACGTCCTGGAAAAAATACAC-3') and introduced in the blunt ended *Nhe*I restriction site, which was provided with T-ends (TA-cloning) and cloned into the pShuttle plasmid of the company Clontech. Thus, the transgene was provided with a hCMV-promoter at the 5'end and with the bovine growth hormone polyadenylation signal at the 3'end. However, it is also within the present invention that E1B55k is used from the plasmid pCGNE1B from Dobbelstein (Dobbelstein, M. et al., EMBO Journal, 16, 4276-4284, 1997) by means of Bam HI and blunt ending and TA-cloning, respectively. The E1B55k-3'UTR which has been cloned, is, among others, described in more detail in Figs. 23 and 24.

Cloning of the vector E4ORF6-IRES-pcDNA3.1(+)

The amplificates E4orf6 using the adenovirus type 5 DNA as template (E4orf6 forward primer 5'-CTTCAGGATCCATGACTACGTCCGGCG-3' and E4orf6 backward primer 5'-GAAGTGAATTCCTACATGGGGGTAGAGTCATAATCGT-3') and from the plasmid pCMVE4-34kD which has been cut with Bam HI (Dobbelstein et al., EMBO, 16, 4276-4284,1997), and the IRES element having the pCITE-4a(+) of the company Novagen as template (IRES forward primer = 5'-TCCGGTTATTTTCCACCATATTGC-3' and IRES backward primer = 5'-TTATCATCGTGTTTTTCAAAGG-3') were subsequently cloned into the multiple cloning site of the pcDNA3.1(+)-vector. For such purpose, primers were used for the E4orf6 transgene which create a *Bam*HI cleavage site at the 5'-end and a *Eco*RI cleavage site at the 3'-end of the open reading frame. The amplificate was digested with the respective restriction enzymes and the ends thereof were made compatible for the directed cloning into the vector which has been opened using *Bam*HI and *Eco*RI. Subsequently, plasmid E4orf6 in

pcDNA3.1(+) was linearized with *Eco*RV, the T-ends added and the amplificate cloned into the IRES element. After checking the correct orientation of the IRES element, the vector was used for further cloning.

The linkage of both transgenes with the IRES element resulted from a cloning of the E4orf6-IRES cassette into the previously generated plasmid CMV-E1B55k 3'UTR-polyA-pShuttle (Clontech) which was linearized with *Not*I, blunt ended and subsequently cut with *Xba*I. E4orf6-IRES in pcDNA3.1 (+) was linearized with *Not*I, the ends made blunt ended and further digested with *Nhe*I. By ligating the E4orf6-IRES insert with the CMV-E1B55k 3'UTR-polyA-pShuttle (Clontech) XVIR-3'UTR was generated in pShuttle (Clontech).

Generation of the used adenoviral shuttle vector

As the shuttle vector pΔE1sp1A, now used for the adenoviral generation system of the company Microbix, did neither contain a CMV promoter nor a bovine growth hormone polyadenylation signal, these elements were cloned into pΔE1sp1A. For such purpose, pΔE1sp1A was linearized with *Cla*I, made blunt ended and cut with *Eco*RI. The element CMV-MCS (multiple cloning site)-poly-A was linearized from pShuttle (Clontech) with MfeI, the ends made blunt ended and further cut with *Eco*RI. Subsequently, the cassette (Xvir-3'UTR pShuttle from Clontech) was cloned with PmeI into the CMV-MCS-poly-A pΔE1sp1A vector which had also been cut with PmeI and subsequently dephosphorylated. The cloning product Xvir-3'UTR- pΔE1sp1A was used for virus generation.

Virus generation

Xvir-3'UTR-pΔE1sp1A and pBHGE3 (from Microbix, contains the E3-region which corresponds to wildtype adenovirus type 5) was cotransfected into HEK 293 cells, whereupon virus Ad-Xvir-3'UTR E3 was generated due to recombination of homologous sequences of both vectors.

Generation of adenovirus Ad-Xvir3'UTR-AdEASY E3 using the AdEASY-system (company Qbiogene)

Generation of the used adenoviral shuttle vector

As, for the present used system, the vector pShuttle-AdEASY did neither contain a CMV-promotor nor the bovine growth hormone polyadenylation signal, these elements were cloned into pShuttle-AdEASY. For such purpose, the plasmid was digested with *Eco*RI, the ends made blunt ended by fling them up with T4-polymerase and dNTPs, the backbone was dephosphorylated and both of the generated digestion products ligated again. By doing so the restriction recognition site for *Eco*RI was eliminated. The thus resulting plasmid was referred to as pShuttle(-*Eco*RI)-AdEASY.

Subsequently, the cassette CMV-MCS-polyA from the pShuttle of Clontech was cut wich M*fe*I and *Eco*RI, the ends made blunt ended and cloned into the vector pShuttle (-*Eco*RI)-AdEASY which was, for such purpose, linearized with *Xba*I, made blunt ended and dephosphorylated. Thus plasmid CMV-MCS-polyA-pShuttle-AdEASY was generated. The cassette E4Orf6-IRES-E1B55k-3'UTR was cloned into this plasmid using *Mlu*I and *Eco*RI. By doing so the plasmid Xvir-3'UTR in pShuttle AdEASY was generated. This was linearized with *Bst*1107I and *Mro*I and introduced into BJ5183 (EC) bacteria together with rescue-plasmid pAdEASY by means of electroporation. By homologous recombination the adenoviral plasmid Ad-Xvir-3'UTR-pAdEASY was generated which resulted in virus production after transfection in HEK293 cells.

Introducing the wt E3 region into pAdEASY

As the E3 region is substantially deleted in plasmid pAdEASY, the E3 region was cloned from plasmid pAdEASY with *Spe*I and *Pac*I into plasmid CMV-MCS-polyA pShuttle (AdEASY) for reconstruction and thus the plasmid E3E4-pShuttle-AdEASY generated.

By restriction with *Nde*I and religation one out of two *Nde*I restriction sites was deleted and so was the multiple cloning site from the plasmid. By this procedure plasmid E3E4-pShuttle(-*Nde*I)-AdEASY was generated.

Subsequently the 4007 bp wtE3-region fragment from wildtype adenovirus type 5 was excised by *Spe*I and *Nde*I and cloned into the E3E4-pShuttle (-*Nde*I)-AdEASY which was opened by *Spe*I and *Nde*I. The thus generated vector was referred to as wtE3E4-pShuttle (*Nde*I)-AdEASY.

Subsequently the wildtype E3E4-region from the E3E4-pShuttle (-*Nde*I)-AdEASY was cut with *Spe*I and *Pac*I and cloned into the pAdEASY and cut with *Spe*I and *Pac*I, whereby in plasmid pAdEASY the E3-region was re-established (pAdEASY-E3). XVir-3'UTR-pAdEASY-E3 was generated by homologous recombination upon transforming BJ5183 (EC) bacteria with plasmids Xvir-3'UTR in pShuttle AdEASY and pAdEASY-E3.

Manipulation of E4 for all of the systems mentioned

In order to provide space for therapeutic transgenes and in order to avoid undesired homologous recombination the E4 region in plasmid E3E4-pShuttle (-*Nde*I)-AdEASY can be deleted specifically. For such purpose, the E4orf6 region is shortened by about 0.6 kB, preferably 629 or 634 bp, by excision with PstI and religation. This can, as described in Fig. 17, be performed in connection with Xvir03/01. Respective deletions are also feasible by the one skilled in the art in different systems for the generation of recombinant adenovirus.

Cloning of the RGD-motif in Ad-Xvir 3'UTR-AdEASY E3 in particular (also applicable to other systems)

For increasing the infectivity the HI Loop of the fibre knob domain was modified following Dmitriev et al. 1998 (An Adenovirus Vector with Genetically Modified Fibers Demonstrates Expanded Tropism via Utilization of a Coxsackievirus and Adenovirus Receptor-Independent Cell Entry Mechanism): The respective region was amplified using the primers RGD-Hpa fw

(5'-GAGgttaacCTAAGCACTGCCAAG-3'), RGD-EcoRV rev (5'-CATAGAGTATGCAGATATCGTTAGTGTTACAGGTTTAGTTTTG-3') and RGD-EcoRV fw (5'-GTAACACTAACGATATCTGCATACTCTATGTCATTTTCATGG-3') and RGD-Bfr rev (5'-CAGCGACATGAActtaagTGAGCTGC-3') and thus an EcoRV restriction site generated. In this restriction site the paired oligonucleotides were cloned which code for an Arg-Gly-Asp (RGD)-peptide: RGD-oligo 1 (5'-CACACTAAACGGTACACAGGAAACAGGAGACACAACTTGTGACTGCCGCGGAGA CTGTTTCTGCCC-3') and RGD-oligo 2 (5'-GGGCAGAAACAG TCTCCGCGGCAGTCA CAAGTTGTGTCTCCTGTTTCCTGTGTACCGTTTAGTGTG-3'). Thus, the RGD motif is present in the HI Loop of the fibre knob domain.

The vector described above is in principle suitable as are the other viruses described herein for use in accordance with the present invention. In particular the afore-described vector is suitable to replicate and trigger lysis insofar, in cells which are YB-1 nucleus-positive cells as well as in cells where YB-1 is deregulated, i. e. is overexpressed compared to normal cells and non-tumor cells, respectively. The use of this vector particularly applies to those diseases and groups of patients or groups of patients which are disclosed in connection with the other adenoviruses which are described herein to be used in accordance with the present invention and the other adenoviruses of the present invention disclosed herein.

Example 12: Structural design of the adenoviral vector Xvir03/01

As may be taken from Fig. 17, Xvir03/01 is a further development of Xvir03. Therapeutic genes such as, for example, the genes described herein and the transgene can be cloned into the E3 region. Additionally, a deletion was introduced into the E4 region so as to avoid homologous recombination with the E4orf6 from the expression cassette of Xvir03. This allows that larger transgenes can be cloned in this construct. The deleted E3 region contains SacI, NdeI and NheI cleavage sites for introducing a cassette, into which, for example, the therapeutic transgenes can be cloned. However, the E3 region may also stay intact and the therapeutic genes may be clines into the E4 region. Thus, among others, the expression of the adenoviral death protein ADP is ensured.

Preparing a plasmid for cloning therapeutic genes into the E3 region as well as for making deletions in the E4 region:

The pAdenoX-Plasmid of Clontech has a restriction site for SfuI behind the 3' ITR region which is absent in wildtype adenovirus. The E3-E4 region was taken from pAdenoX (Clontech) with the SpeI (position 23644) and SfuI and transferred into pcDNA3.1(+) (Invitrogen) = pcDNA3.1-E3Δ27865-30995-E4. The bigger part of E4ORF6, namely 33241-33875 was removed by means of PstI = pcDNA3.1-E3Δ27865-30995,E4Δ33241-33875. For the further development of Xvir03 the deleted E3/E4 region from pcDNA3.1-E3Δ27865-30995,E4Δ33241-33875 was cloned by means of SfuI and SpeI into plasmid pAdenoX = pAdenoX E3Δ27865-30995,E4Δ33241-33875.

The expression cassette was subsequently, as described for Xvir03, cloned with I-Ceu I and PI-SceI from the E1B55k-IRES-E4orf6-pShuttle together with the CMV promoter and the *bovine growth hormone* (BGH)-PolyA into pAdenoX E3Δ27865-30995,E4Δ33241-33875 and referred to as AdcmvE1B/IRES/E4orf6-ΔE4. Subsequently, the adenovirus was prepared in accordance with manufacturer's instructions (Clontech).

It is within the present invention and feasible for he one skilled in the art in the light of the present disclosure that other systems nay be used for the manufacture of the adenoviruses in accordance with the present invention and in particular the recombinant adenoviruses, such as the systems of the companies QBIOGENE and Nicrobix.

The afore-described vector is in principle useful as are the other viruses described herein to be used in accordance with the present invention. In particular the afore-described vector is suitable to replicate in YB-1 nucleus-positive cells as well as cells in which YB-1 is deregulated, i. e. is overexpressed compared to normal cells and non-tumor cells, and to cause lysis insofar. This vector can also be used for those diseases and groups of patients and collectives of patients which are disclosed herein for the other adenoviruses to be used in accordance with the present invention and the adenoviruses in accordance with the present invention.

Example 13: Oncolytic effect of Xvir 03 in 257 RDB and 181 RDB cells

100,000 cells (257RDB and 181RDB) were plated per well of a plate having six wells (engl.: 6 well plate). AT the next day the cells were, as depicted in Fig. 18, infected with Ad312 (20 pfu/cell) and Xvir03 (5 pfu/cell). The infection was performed in 500 µl serum free DMEM medium at 37° C for 1 h. Subsequently, the infection medium was removed and replaced by 2 ml complete medium (10 % FCS/DMEM). The analysis was done by means of crystal violet staining after 5 days. The result is depicted in Figs. 18A and 18B.

As may be taken from Fig. 18A and 18B, the multidrug resistant cells which have YB-1 in the nucleus, show lysis after infection with Ad312 and Xvir03 only in case of Xvir03 as represented by the crystal violet staining of the cells. In connection therewith, first the medium is removed. Subsequently the cells are covered with crystal violet (50 % ETOH, 3 % formaldehyde, 5 % acetic acid, 1 % crystal violet) and incubated at room temperature for 5 – 10 min. Subsequently, the six well plates are thoroughly rinsed with water and dried at room temperature.

It is known to the present inventor that E1A-deleted viruses (e. g. Ad312) which, however, are not transactivating adenoviruses in the sense of the present invention, may replicate very efficiently at higher MOIs (Nevins J. R., Cell 26, 213-220, 1981), which, however, cannot be realised in clinical application. This phenomenon is referred to in the literature as "E1A-like activity". The adenovirus Ad312 as used herein, is an E1A-deleted virus. At the titer used (20 pfu/cell), which is still above the clinically desirable titer, the early adenoviral genes such as E1B55k and E4orf6 are not expressed or expressed only to a very small extent (Nevins J. R., Cell 26, 213-220, 1981). As already described herein, these genes and proteins play an important role in viral replication. In contrast thereto, these genes and proteins, respectively, are expressed by adenovirus Xvir03 (Figs. 16). As may be taken from Fig. 18A and 18B, the expression of the genes E1B55k and E4orf6 will result in an efficient viral replication and cell lysis at a concomitantly lower infection titer required (expressed as pfu/cell). This confirms the finding underlying the present invention, namely that the expression of E4orf6 and E1B-55K (and the absence of E1A) in combination with nuclear localisation of YB-1 is capable of inducing a very efficient adenoviral replication. The titer required therefor of only 1 to 5 pfu/cell now allows for clinical application.

This confirms the finding underlying the present invention, namely that the presence of YB-1 in the nucleus, particularly the presence independent from the cell cycle, is required in order to make the viruses which are to be used in accordance with the present invention, lyse infected cells.

Example 14: Replication of adenovirus in cells after addition of Irinotecan

In order to determine the effect of Irinotecan on adenoviral replication 10^6 U373 tumour cells were plated in 10 cm^2 Petri dishes. In a first reaction 5µM Irinotecan was added after 24 hours. After another 24 hours the cells were infected with 10 pfu/cell dl520. After incubation of 3 days without Irinotecan DNA was isolated in accordance with the procedure described in example 10.

In a parallel reaction the thus prepared U373-cells were not pre-incubated with Irinotecan. After 48 hours of cultivating the cells without Irinotecan, they were infected with 10 pfu/cell dl520 and subsequently incubated without Irinotecan for another 3 days. DNA was isolated as described above.

Subsequently 2 µ DNA were digested with restriction enzyme Kpn I and a Southern Blot analysis performed. A part of the adenoviral genome (position:22734-24235) generated by means of PCR was used as a probe.

The result is depicted in Fig. 19. Fig. 19 shows that after incubation with Irinotecan adenoviral replication is significantly increased in U373 cells after treatment with Irinotecan (lane 2) compared to untreated control where no incubation with Irinotecan was performed (lane 1). This means that adenoviral replication is increased under the influence of Irinotecan.

Example 15: Replication of adenovirus in cells after administration of Trichostatin A

In order to test the effect of Trichostatin A on adenoviral replication, 10^6 U373 tumour cells were plated in 10 cm^2 Petri dishes. After 24 hours 0, 0.25, 0.5 and 0.75 µM Trichostatin A was added. After another 24 hours the cells were infected with 10 pfu dl520/cell.

After 3 days of incubation in medium without Trichostatin DNA was isolated. Subsequently 2 µg DNA were digested with restriction enzyme Kpn I and a Southern Blot analysis performed. A part of the adenoviral genome (position:22734-24235) generated by means of PCR was used as a probe.

The result is depicted in Fig. 20. Fig. 20 shows that after incubation with increasing concentrations of Trichostatin A viral replication in U373 cells (lanes 2, 3 and 4) is significantly increased compared to untreated controls where no incubation with Trichostatin A was performed (lane 1). This means that viral replication is increased under the influence of Trichostatin A.

Example 16: Influencing the expression of Coxsackievirus-Adenovirus-receptor (CAR) on U373 cells in response to addition of Trichostatin A

200,000 U373 cells were plated in 6 well plates. After 24 hours the cells were cultivated with 1 µM Trichostatin for 24 hours. After another 24 hours the cells were isolated. Subsequently, analysis of CAR expression was performed according to a standard protocol using Facs-analysis and the primary antibody anti-CAR clone RmcB from the company Upstate, and a rabbit anti-mouse FITC as secondary antibody (company DAKO).

The result is depicted in Fig. 21. Without Trichostatin treatment 11.3% of the cells were CAR-positive, whereas after incubation of the cells with 1μM Trichostatin 56.2% of the cells were CAR-positive. The figures are percentages of the overall cells used in the test.

From Fig. 21 it can be taken that under the influence of the histone deacylase inhibitor Trichostatin A CAR, which is an important factor for the binding of adenovirus, is expressed at a higher level and is more available, respectively, which increases the efficacy of transfection of the thus treated cells.

Example 17: Oncolysis of U373 cells by adenovirus after combined treatment of the cells with Irinotecan and Trichostatin A.

200,000 U373 cells were plated in a 6 well plate. After 24 hours either 2 μM Irinotecan or only 1 μM Trichostatin A or 1 μM Irinotecan + 0.5 μM Trichostatin were added to the medium. After 24 hours of incubation the cells were infected with 10, 20 and 30 pfu/cell dl520. After 3 – 5 days the analysis was performed using crystal violet staining. The assays were performed in duplicate.

The result is depicted in Fig. 22. The six plates represented in panel 1 show a complete cell layer which was not affected by incubation with a combination of Irinotecan and Trichostatin A as shown by crystal violet staining. The next two wells of panel 1 show the cell layer after infection with 10 and 20 pfu/cell dl520, respectively. Also under such conditions there is no lysis of the cells which is due to the absence of replication of dl520. Thus it is shown that neither dl520 at 10 or 20 pfu/cells nor 1 μM Irinotecan + 0.5 μM Trichostatin A alone are able to induce cell lysis.

The further 6 well plates 2, 3 and 4 depicted in Fig. 22, herein also referred to as panels 2, 3 and 4, were basically treated in accordance with the foolowing scheme. The individual wells were inoculated with U373 cells as previously described and the cells cultivated therein. The wells were inoculated with 10, 20 or 30 pfu/cell dl520 in duplicate, whereby the differences

between the three 6 well plates resided in the kind of cytostatics used. In panel 2 2 μM Irinotecan, in panel 3 1 μM Trichostatin A and in panel 4 1 μM Irinotecan and 0.5 μM Trichostatin A was added to the individual plates.

In the 6 well plate 2 (panel 2) with 2 μM Irinotecan the cells were lysed with 30 pfu/cell dl520. In the 6 well plate 3 (panel 3) with 1 μM Trichostatin A the cells were lysed at 20 and 30 pfu/cell dl520. In the 6 well plate 4 (panel 4) with 1 μM Irenotecan + 0.5 μM Trichostatin A the cells, in contrast thereto, were already lysed at 10 pfu/cell dl 520.

The test, the results of which are depicted in Figs. 19 to 23, shows that the combination consisting of Irinotecan + Trichostatin A + dl520 induces a more effective cell lyses of tumour cells as any compound alone. This results, on the one hand, from Trichostatin A increasing CAR-expression and thus significantly improves infectability of the cells. On the other hand, Irinotecan translocates YB-1 into the cell nucleus and thus induces an improved adenoviral replication. Additionally, the cellular YB-1 is assisting adenoviral replication after infection with dl520 and is no longer available for DNA-repair processes. Depending on the point of view, this results in an improved efficacy of dl520 on the one hand and an increased efficacy of the cytostatics on the other hand.

The features of the invention disclosed in the preceding specification, the claims as well as the figures can both individually as well as in any combination be important to the realisation of the invention in its various embodiments.